Generation of antigen-specific T cell immunity through T cell receptor gene transfer

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Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit van Leiden,

op gezag van Rector Magnificus prof. mr. P.F. Van der Heijden,

volgens het besluit van het College voor Promoties

te verdedigen op woensdag 21 januari 2009

klokke 16.15 uur

door

Miriam Coccoris

geboren te Haarlem

in 1977

Promotiecommissie:

Promotor:	Prof. Dr. T.N.M. Schumacher
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The studies described in this thesis were performed at the Department of Immunology at the Netherlands Cancer Institute (NKI), Amsterdam.

This study was financially supported by grants from the Netherlands Organization for Scientific Research (NWO Pioneer grant 00-03) and the EU 6th Framework Program (ATTACK).

The printing of this thesis was financially supported by: VIP Aviation, de specialist in Air & Ocean Recruitment.

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Scope of this thesis

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T cells defend our body against invading pathogens such as bacteria, viruses, fungi and parasites. Fragments of these pathogens are presented to the T cell through the Major Histocompatibility Complex (MHC), which is present on most cell types. T cells recognize the presented peptide fragments as foreign via their T cell receptor (TCR) and kill the infected host cell. Due to selection processes in the development of T cells, the majority of self-reactive T cells is deleted from the repertoire (tolerance), providing us with a powerful defence against invading organisms without harmful reactivity towards uninfected healthy cells.

A drawback of this elegant system is that due to this tolerance high-affinity tumor-specific T cells are often absent from the repertoire, rendering it generally unresponsive to active vaccination. As tumor associated antigens (TAA), which are potential targets for immunotherapy of cancer, mostly consist of non-mutated lineage specific- or aberrantly expressed self-antigens, they make poor targets for boosting existing immunity because of the previously mentioned tolerance. In cases when active immunization is not applicable, passive immunization by means of the adoptive transfer of tumor-specific T cells can offer a solution. Currently, adoptive immunotherapy approaches rely on the presence of tumor infiltrating lymphocytes (TIL) in patients, which can be isolated from resected tumor tissue and can be expanded *ex vivo*. However, this is a laborious process, which is patient-restricted and the recovery of TIL with clear tumor-specificity is only possible in a small subset of patients.

The manipulation of T cells by introducing TCR genes of interest through retroviral gene transfer can overcome this problem. The technique of TCR gene transfer allows for the creation of T cells that are equipped with a tumor-specific TCR, thus making it possible to generate tumor-specific T cells from pre-existing autologous T cells. This technique is patient-tailored and relatively fast, making it an attractive off-the-shelf alternative for existing immunotherapy for the treatment of cancer, and moreover yields the advantage to create T cells with specificities which are normally absent in patients. In this thesis the main goal has been to explore the possibilities and limitations of TCR gene therapy in different mouse models. We have explored the feasibility of adoptive transfer of TCR gene modified T cells targeting a self-/tumor- antigen *in vivo* and have investigated the safety of TCR transduced T cells in mice of different MHC class I background (chapter 3). Adding to that we have investigated the persistence of adoptively transferred TCR modified T cells *in vivo* and have looked at the capacity of T cells equipped with a virus-specific TCR to form T cell memory

(chapter 4). We have also investigated an alternative strategy for TCR gene transfer that aims to prevent the formation of mixed dimers consisting of endogenous and exogenous TCR chains that can occur after introduction of the TCR genes into alpha-beta T cells. To this purpose we have introduced TCR genes into gamma-delta T cells instead of alpha-beta T cells and we have documented the capacity of such TCR-modified gamma-delta T cells to function *in vivo* (chapter 5).

Finally, we have developed a new technology to identify T cell epitopes in a high-throughput format. This technology may enable the identification of novel candidate tumor-antigens recognized by T cells. The development and validation of this technology are described in chapter 2 of this thesis.

Chapter 1

Introduction:
Prospects and limitations of T cell receptor gene therapy

Miriam Coccoris, Moniek A. de Witte and Ton N.M. Schumacher

Current Gene Therapy 5: 583-593 (2005)

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-

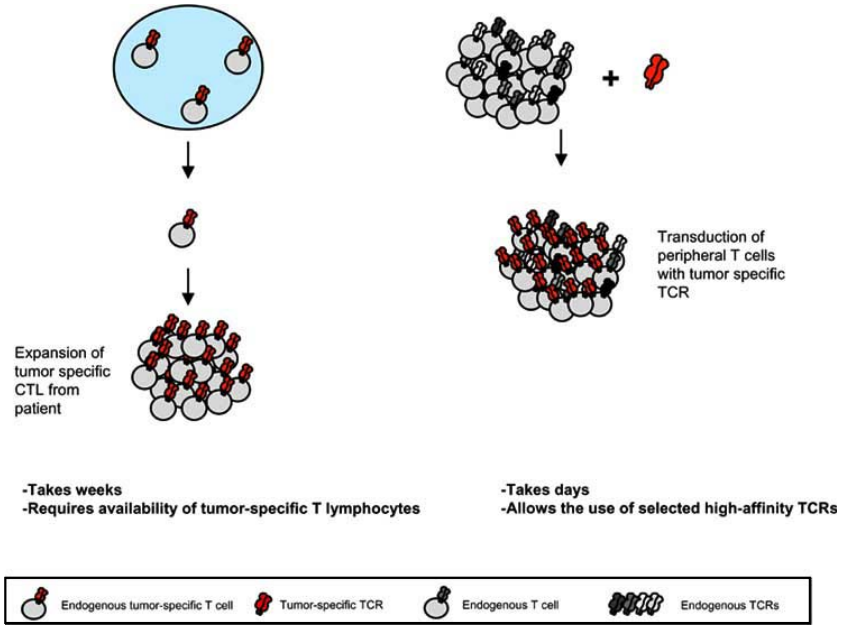

Fig. (I). Adoptive transfer of T cells and T cell receptors. Potential advantages of TCR gene transfer over conventional cellular transfer. Left: Immunotherapy *via* adoptive transfer of T cells. This approach has two major limitations. First, tumor specific T cells can only be isolated from a subset 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.

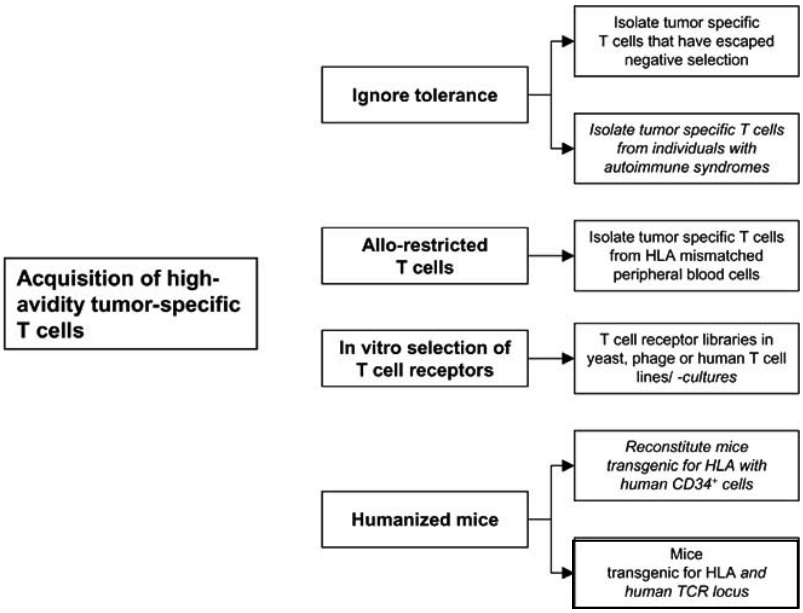

Fig. (2). Acquiring high avidity tumor-specific T cell receptors. To generate collections of T cell receptors that recognize self-antigens with high affinity, several strategies have been used (regular typing) or may be proposed (italic typing). References for the previously used approaches for obtaining 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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Received: April 29, 05 Revised: July 13, 2005 Accepted: September 07, 2005

Chapter 2

Design and use of conditional MHC class I ligands

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Nature Medicine 12: 246-251 (2006)

Design and use of conditional MHC class I ligands

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Major histocompatibility complex (MHC) class I molecules associate with a variety of peptide ligands during biosynthesis and present these ligands on the cell surface for recognition by cytotoxic T cells. We have designed conditional MHC ligands that form stable complexes with MHC molecules but degrade on command, by exposure to a defined photostimulus. 'Empty MHC molecules' generated in this manner can be loaded with arrays of peptide ligands to determine MHC binding properties and to monitor antigen-specific T-cell responses in a high-throughput manner. We document the value of this approach by identifying cytotoxic T-cell epitopes within the H5N1 influenza A/Vietnam/1194/04 genome.

MHC class I molecules are heterotrimers that consist of an invariant light chain, a polymorphic heavy chain and an 8–10-amino-acid peptide ligand. The peptide forms an essential subunit of the MHC class I complex, as MHC class I molecules that do not associate with peptide ligand are unstable[1,2]. Association of peptides with MHC class I molecules is in large part based on shape and electrostatic complementarity between two amino-acid side chains at the anchor positions of the peptide and MHC allele–specific pockets[3,4]. In addition, binding of peptide ligands depends on the interaction of the MHC molecule with the terminal α-amino and carboxyl groups of the peptide[5,6]. Because the sequence requirements for binding to MHC are largely restricted to two dominant anchor residues[7], the average protein contains several dozen potential T-cell epitopes and, as an example, the approximately 100 open reading frames of a member of the herpesviridae family contain an estimated 4,000 potential T-cell epitopes. Definition of tumor-and pathogen-encoded MHC ligands and detection of T-cell responses specific for such ligands remains a major challenge.

The visualization of antigen-specific T-cell responses was first made possible with the development of tetrameric MHC reagents[8]. In this strategy, soluble MHC monomers complexed with a peptide of interest are biotinylated and converted to tetravalent structures by binding to fluorochrome-conjugated streptavidin or avidin. The resulting MHC tetramers have become essential reagents for the detection of antigen-specific $CD4^+$ and $CD8^+$ T cells by flow cytometry. More recent work indicates that characterization of antigen-specific T-cell responses by MHC microarray–based strategies is also feasible[9,10]. Furthermore, MHC-based selection of antigen-specific T cells has been proposed as a strategy to boost melanoma-specific T-cell responses in individuals with melanoma[11] and to provide defined minor histocompatibility antigen–specific and virus-specific T cells to recipients of allogeneic stem cell transplants and other immunocompromised individuals[12–17]. At present, the major limitation of MHC tetramer–based technologies is the involved and lengthy nature of the refolding and purification steps required to generate every individual peptide-MHC complex. Consequently, it has not been feasible to apply MHC tetramer–based technology for high-throughput applications. To address this issue, we have explored the possibility of identifying conditional MHC ligands that can be used to generate peptide-receptive MHC class I molecules at will.

RESULTS

Design of conditional MHC class I ligands

We set out to create MHC class I ligands that would disintegrate on command while bound to the MHC complex. As a building block for such conditional ligands, we synthesized a 9-fluorenylmethyloxycarbonyl (Fmoc)-derivative of 3-amino-3-(2-nitro)phenyl-propionic acid (abbreviated 'J' in the amino acid sequence). Subsequently, we used this building block to generate variants of the human leukocyte antigen (HLA)-A2.1–restricted influenza A matrix 1 (M1)(58–66) epitope (sequence, GILGFVFTL). An example of such a variant, in which Thr8 is replaced by 3-amino-3-(2-nitro)phenyl-propionic acid, is compound I (sequence, GILGFVFJL; **Fig. 1a**). Consistent with published literature[18,19], this compound disintegrates upon exposure to ultraviolet (UV) light (**Fig. 1a**), and after exposure to UV-light, a molecule with a mass-to-charge ratio of 751.4 becomes apparent, corresponding to the mass of the heptameric peptide acetamide fragment (compound II; **Fig. 1**). We did not study further the small, C-terminal peptide fragment that is also generated during this cleavage reaction.

To establish whether UV light–sensitive ligands can be used to generate peptide-MHC complexes, we used two influenza A M1(58–66) variants with 3-amino-3-(2-nitro)phenyl-propionic acid incorporated at position 4 (compound III; GILJFVFTL; **Supplementary Fig. 1** online) or position 8 (compound I; **Fig. 1a**) in MHC class I refolding reactions[20]. MHC class I refolding reactions with either GILJFVFTL or GILGFVFJL produced high yields of HLA-A2.1–peptide complexes. As

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Received 11 November 2005; accepted 13 December 2005; published online 5 February 2006; doi:10.1038/nm1360

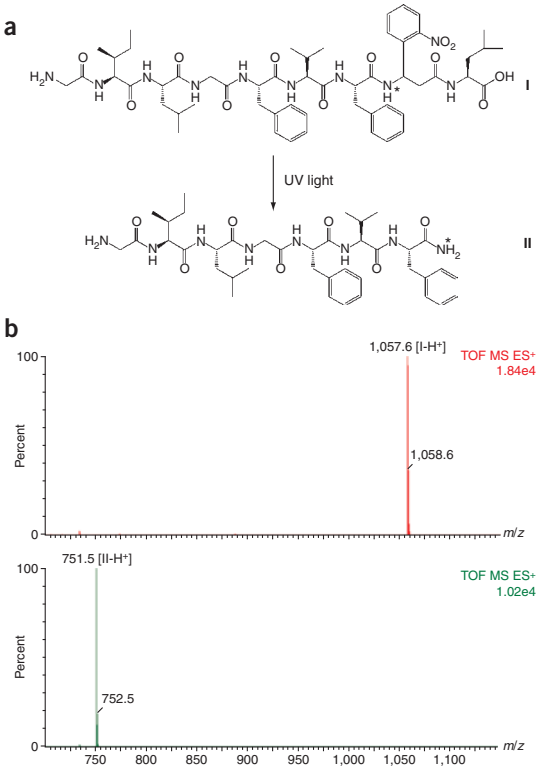

Figure 1 Photocleavage strategy. (**a**) Structure of compound I (top), the photocleavable analog of the influenza A M1(58–66) epitope obtained as an approximate one-to-one mixture of diastereoisomers. Amino acid sequence, GILGFVFJL. Structure of compound II (bottom), the heptameric peptide fragment generated upon UV light–induced degradation of compound I. Asterisks in compounds I and II indicate the preferred cleavage site. (**b**) Liquid chromatography–mass spectrometry of compound I before (top) and after (bottom) exposure to 366-nm light for 60 min, in the presence of 0.5 mM DTT. Expected masses: single-protonated compound I: 1,057.6; single-protonated compound II: 751.4. Numbers in the upper right corner reflect the total ion counts in time-of-flight positive electrospray ionization mode.

a first crude test of the sensitivity of the non-natural MHC-bound ligands to UV light–mediated cleavage, we exposed HLA-A2.1–peptide complexes containing either the unmodified influenza A M1(58–66) epitope, or the GILJFVFTL or GILGFVFJL variant to UV light and analyzed the reaction products by gel-filtration chromatography. Whereas the unmodified HLA-A2.1–peptide complex was not affected by exposure to 366-nm light, exposure of either HLA-A2.1–GILJFVFTL (data not shown) or HLA-A2.1–GILGFVFJL (**Supplementary Fig. 2** online) resulted in a substantial reduction in recovery of MHC molecules. Furthermore, when we prepared HLA-A2.1–peptide complexes with an influenza A M1(58–66) derivative containing the UV light–resistant building block 3-amino-3-phenyl-propionic acid[21], the resulting MHC complexes were insensitive to exposure to UV-light, showing that the UV light–induced decay of HLA-A2.1–GILJFVFTL and HLA-A2.1–GILGFVFJL requires the presence of the nitrophenyl moiety. Notably, the observed decrease in the amount of folded MHC upon exposure to UV light of the HLA-A2.1–GILGFVFJL complex can be prevented by inclusion of HLA-A2.1–binding peptides during the cleavage reaction, but not by inclusion of a control HLA-A3–binding peptide (**Supplementary Fig. 2** online), suggesting efficient peptide exchange.

Although these data indicate the sensitivity to UV light of HLA complexes containing 3-amino-3-(2-nitro)phenyl-propionic acid–based peptide ligands, it is difficult to establish the efficiency of this cleavage reaction by gel-filtration chromatography (**Supplementary Fig. 2** online). As a more stringent test for replacement of the conditional ligand by the newly added peptide, we exposed HLA-A2.1–GILGFVFJL complexes to UV light in the presence of the cytomegalovirus (CMV) pp65(495–503) epitope, and after this reaction, we purified the peptide–MHC complexes and analyzed them by mass spectrometry. The major peptide mass that is visible before exposure to UV light corresponds to the Na^+ ion of GILGFVFJL, providing formal proof that this ligand forms a stable complex with HLA-A2.1 (**Fig. 2**). After UV light–mediated cleavage, no detectable amount of GILGFVFJL remains associated with HLA-A2.1. Furthermore, the amount of compound II complexed with HLA-A2.1 is less than background, suggesting that dissociation of this heptameric peptide fragment is essentially complete. Instead, upon UV light–mediated cleavage the sole detectable peptide mass associated with

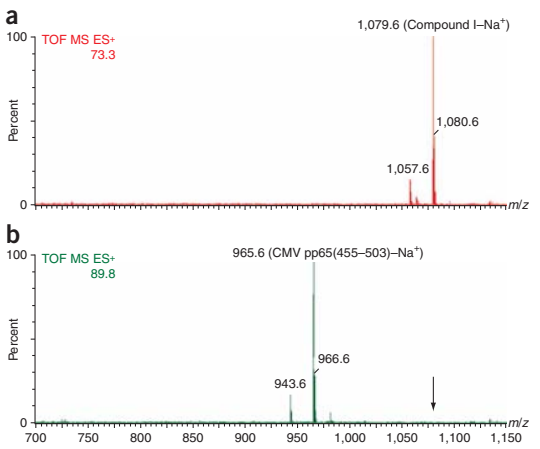

Figure 2 UV light–mediated peptide exchange. Mass spectrometric analysis of HLA-A2.1–associated peptide of HLA-A2.1–GILGFVFJL complexes before (top) and after (bottom) exposure to UV light in the presence of CMV pp65(495–503) peptide. Observed mass-to-charge ratios and assignments are indicated. Expected masses: Na^+ ion of compound I: 1,079.6; Na^+ ion of CMV pp65(495–503): 965.5. There was a lack of detectable compound I–Na^+ after UV light–induced peptide exchange (arrow). We exposed 25 μM HLA-A2.1–GILGFVFJL in 20 mM Tris-HCl (pH 7.0), 150 mM NaCl and 0.5 mM DTT to 366-nm light for 1 h on ice in the presence of 500 μM CMV pp65(495–503) peptide. Subsequently, HLA-A2.1-peptide complexes were purified by gel-filtration chromatography on a Biosep SEC S-3000 column in 25 mM NH_4OAc (pH 7.0) and directly used for analysis on a Waters LCT ESI mass spectrometer by direct infusion under optimized conditions (160 V cone voltage, 160 °C desolvation temperature). The use of high cone voltage and high temperature resulted in the detection of predominantly sodiated species. Numbers in the upper right corner reflect the total ion counts in time-of-flight positive electrospray ionization mode.

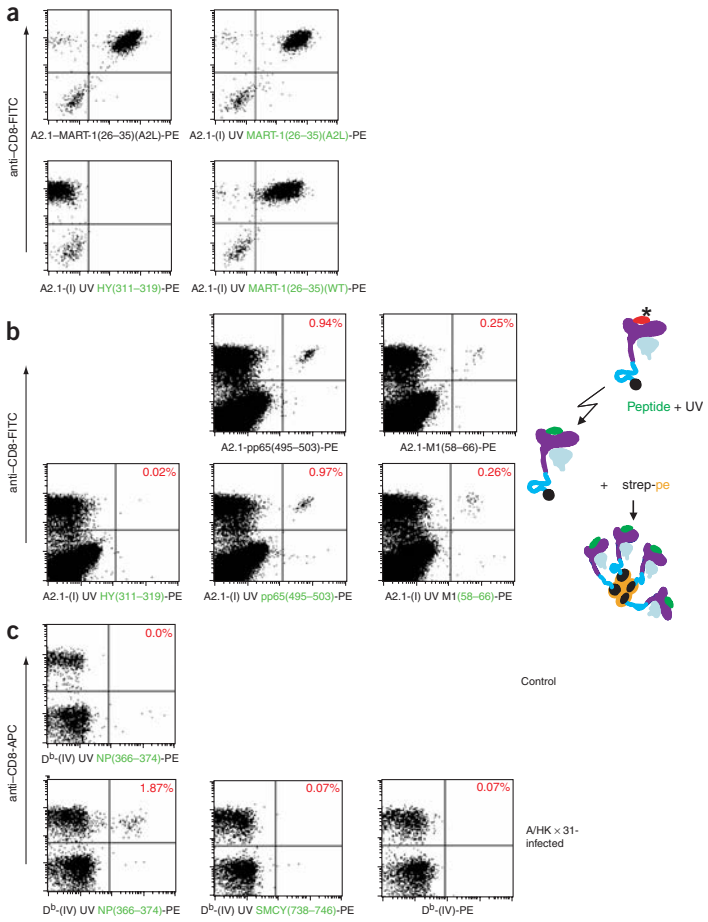

Figure 3 T-cell staining with MHC exchange tetramers. (**a**) Flow cytometric analysis of MHC tetramer staining of a MART-1(26–35)-specific CTL clone with classical MHC tetramers containing the MART-1(26–35)(A2L) epitope (top left), or MHC exchange tetramers containing the MART-1(26–35)(A2L) epitope (top right), a control peptide HY(311–319) (bottom left), or the naturally occurring low-affinity MART-1(26–35) epitope (bottom right). (**b**) Flow cytometric analysis of peripheral blood mononuclear cells from an HLA-A2.1–positive individual stained with classical MHC tetramers containing the CMV pp65(495–503) epitope (top left) or influenza A M1(58–66) epitope (top right), or with MHC exchange tetramers containing a control peptide HY(311–319) (bottom left), the CMV pp65(495–503) epitope (bottom middle) or the influenza A M1(58–66) epitope (bottom right). (**c**) Flow cytometric analysis of MHC tetramer staining of peripheral blood mononuclear cells of a C57BL/6 mouse (top) or a C57BL/6 mouse 8 d after intranasal infection with influenza A/HKx31, encoding the A/PR8/34 NP(366–374) ASNENMETM epitope (bottom). Analysis was performed with H-2D^b exchange tetramers containing the A/PR8/34 NP(366–374) epitope (left), a control peptide (SMCY(738–746) bottom middle), or H-2D^b tetramers prepared from biotinylated H-2D^b–ASNENJETM monomers that had not undergone exchange reactions (bottom right). Cartoon depicts the process used to generate MHC exchange tetramers. The UV light–sensitive peptide is indicated by an asterisk. I and IV refer to the UV light–sensitive ligands for HLA A2.1 and H2-D^b.

HLA-A2.1 corresponds to the mass of the CMV pp65(495–503) epitope (**Fig. 2**). Collectively, these experiments indicate that we can produce conditional MHC ligands that are released from MHC molecules upon exposure to UV light and that MHC molecules generated in this process can be loaded with epitopes of choice. To extend these data to other conditional HLA-A2.1 ligands, we synthesized a peptide that is predicted to bind avidly to HLA-A2.1 (ref. 22), with the UV light–sensitive building block J incorporated at position 8 (ILAETVAJV). Refolding reactions with this conditional ligand gave high yields of folded HLA-A2.1–peptide complexes and, analogous to the data obtained with HLA-A2.1–GILGFVFJL complexes, these complexes disintegrated after exposure to UV light (data not shown).

MHC exchange tetramers

To test the potential value of this MHC exchange technique for the visualization of antigen-specific T cells, we performed MHC exchange reactions with biotinylated HLA-A.2.1– GILGFVFJL or HLA-A.2.1–ILAETVAJV complexes. Then we added phycoerythrin-streptavidin and used the resulting MHC tetramers (hereafter referred to as MHC exchange tetramers) to detect antigen-specific T cells by flow cytometry. MHC exchange tetramers containing the high-affinity (A2L) variant of the Melan-A/MART-1(26–35) epitope[23] stain a MART-1–specific cytotoxic T lymphocyte (CTL) clone as efficiently as conventional MHC class I tetramers (**Fig. 3a**). Likewise, MHC exchange tetramers can be used to detect low-magnitude T-cell responses in peripheral blood samples (**Fig. 3b** and **Supplementary Fig. 3** online). UV light–induced cleavage of MHC-bound ligands also allows the synthesis and use of MHC tetramers that contain the naturally occurring MART-1(26–35) peptide that binds to MHC class I molecules with low affinity, because of the absence of a leucine or methionine residue at the anchor site at position 2 (**Fig. 3a**). This indicates that UV light–induced peptide exchange is sufficiently robust to screen collections of putative T-cell epitopes without *a priori* knowledge of MHC binding affinities. Notably, MHC tetramers prepared from HLA-A2.1–GILGFVFJL complexes or A2K^b-GILGFVFJL complexes (in which the HLA-2.1 α3 domain is replaced by the mouse MHC H-2K^b α3 domain) that have not been exposed to UV light, and that are therefore uniformly occupied by the UV light–sensitive influenza A M1(58–66) epitope variant, do not stain polyclonal influenza A M1(58–66) epitope–specific T cells (**Supplementary Fig. 3** online). This indicates that either the alteration in the peptide backbone as a result of the introduction of an unnatural β-amino acid, or the replacement of the threonine side chain is incompatible with T-cell recognition by M1(58–66)–specific T cells. Consequently, even in settings in which the release of conditional ligand would not be

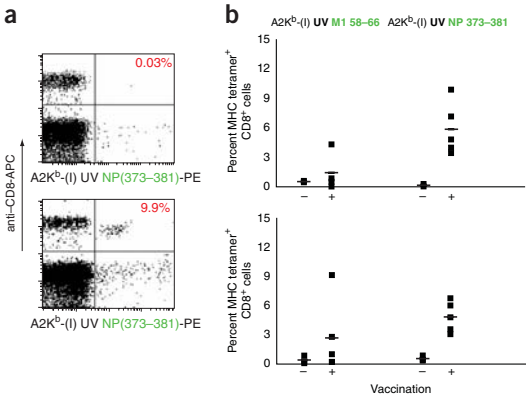

Figure 4 High-throughput screen of H5N1 T-cell epitopes. (**a**) Flow cytometric analysis of mouse peripheral blood mononuclear cells stained with A2K^b-NP(373–381) exchange tetramers of either a nonvaccinated HLA-A2.1 transgenic mouse (top) or of an HLA-A2.1 transgenic mouse (bottom) vaccinated at days 0, 3 and 6 with 20 μg of influenza A/Vietnam/1194/04 NP-encoding DNA. (**b**) Top, T-cell responses of individual mice (closed squares) and average T-cell responses (stripes) of nonvaccinated mice (–) and mice vaccinated with the influenza A/Vietnam/1194/04 M1 (left, +) and NP (right, +) genes, analyzed with the indicated A2K^b exchange tetramers at day 13 after primary vaccination. At the peak of the vaccination-induced T-cell response, peripheral blood was drawn. Blood of mice in each of the four groups was pooled and analyzed by MHC tetramer staining. MHC tetramers that scored positive were used for reanalysis of individual mice. Bottom, T-cell responses of individual mice (closed squares) and average T-cell responses (stripes) of nonvaccinated mice (–) and mice vaccinated with the influenza A/Vietnam/1194/04 M1 (left, +) and NP (right, +) genes, analyzed with the indicated A2K^b exchange tetramers at day 11 after secondary vaccination. The identity of the NP(373–381) epitope was confirmed by synthesis of this sequence on a preparative scale followed by high-performance liquid chromatography purification and screening of vaccinated mice by MHC exchange tetramer staining and intracellular interferon-γ staining (data not shown). NP, nucleoprotein; M1, matrix protein 1.

optimal, MHC exchange reagents are not expected to have background reactivity because of the presence of residual conditional ligand. Should conditional ligand-MHC complexes for other alleles display background reactivity, it should be straightforward to prevent such reactivity by modification of T-cell receptor–exposed side chains. Notably, MHC tetramers generated by UV light–mediated exchange compare favorably with MHC-immunoglobulin dimers generated by passive peptide exchange[24,25], both with respect to signal intensity and signal-to-noise ratios (**Supplementary Fig. 4** online).

To test whether conditional ligands may readily be identified for other MHC class I alleles, we synthesized four variants of the influenza A NP(366–374) epitope (sequence, ASNENMETM) that is presented by the mouse MHC class I allele H-2D^b. Two of those variants, IV and V (**Supplementary Fig. 1** online), fulfilled both criteria in that H2-D^b complexes could be generated with these conditional ligands and that these ligands could be cleaved in the MHC-bound state. Consistent with the data obtained for HLA-A2.1, H-2D^b exchange tetramers prepared from UV light–sensitive H-2D^b–ASNENJETM complexes stained antigen-specific T cells with high specificity (**Fig. 3c**). Furthermore, as is the case for HLA-A2.1–GILGFVFJL tetramers, H-2D^b–ASNENJETM tetramers that are uniformly occupied by the UV light–sensitive variant of NP(366–374) did not stain influenza A NP(366–374)–specific T cells (**Fig. 3c**).

High-throughput screening with MHC exchange reagents

As a first test of the potential of MHC exchange for high-throughput epitope mapping, we cloned and sequenced the four genes encoding the immunodominant proteins of influenza A/Vietnam/1194/04, an influenza A H5N1 strain isolated from an individual with a fatal case of influenza in Vietnam, and scanned the encoded proteins for potential HLA-A2.1–binding peptides. Within the genes encoding hemagglutinin, neuraminidase, M1 and nucleoprotein (NP), we identified 132 potential epitopes with a score for predicted MHC binding of $\geq$20 (ref. 22), and these peptides were produced by microscale synthesis. In parallel, we used the same set of genes to prepare vectors for DNA vaccination and vaccinated groups of HLA-A2.1 transgenic mice[26] by DNA tattoo[27]. At the peak of the vaccination-induced T-cell response, we generated a collection of MHC exchange tetramers by performing 132 parallel UV light–mediated exchange reactions on A2K^b-GILGFVFJL complexes[28] in microtiter format, and used the resulting MHC tetramer collection to screen peripheral blood samples of vaccinated mice. This analysis showed the presence of two T-cell epitopes within these four gene products of influenza A/Vietnam/1194/04. Specifically, this screen confirmed the immunogenicity of the known influenza A M1(58–66) epitope that is conserved between the majority of influenza A strains. In addition, this scan indicated the presence of a previously unknown HLA-A2.1–restricted T-cell epitope located in the influenza A/Vietnam/1194/04 NP (**Fig. 4**). Notably, in this HLA-A2.1 transgenic mouse model, the immunogenicity of this epitope (NP(373–381)) is substantially higher than that of the classical influenza A M1(58–66) epitope (M1(58–66), one out of five responding mice after primary vaccination, three out of five responding mice after secondary vaccination; NP(373–381), five out of five responding mice after primary vaccination). This previously unknown T-cell epitope is shared between H5N1 strains of the past years but is distinct in older influenza A strains.

DISCUSSION

Here we have described conditional MHC ligands that can disintegrate in the MHC-bound state under conditions that do not affect the integrity of the MHC molecule, thereby permitting the reloading of assembled MHC molecules with epitopes of choice. The peptide ligands that are bound to the MHC after exchange are not modified, nor is the MHC backbone altered. In line with this, MHC complexes generated by this exchange technology have the predicted binding specificity in all cases tested. This strategy and related chemical cleavage strategies should be of substantial use in the high-throughput identification of both MHC ligands and cytotoxic T-cell responses. In this strategy, a single batch of UV light–sensitive MHC complex is prepared by the classical *in vitro* MHC class I refolding and purification protocols, and this UV light–sensitive MHC complex is subsequently used to generate large arrays of desired peptide-MHC complexes in 1-h exchange reactions.

MHC-immunoglobulin dimers purified from eukaryotic cells have previously been used to generate peptide-MHC reagents, by performing exchange reactions with exogenously added peptide[24,25]. Although the overall goal of this technology is similar to that of UV light–induced peptide exchange, the technologies differ at essential points. Specifically, whereas the MHC-immunoglobulin dimer technology

depends on the slow release of a pool of unknown endogenous peptides and is facilitated by conditions (for example, low or high pH) that also destabilize the MHC molecule, UV light–induced peptide exchange is based on the release of a single ligand, by exposure to a defined trigger that does not affect the integrity of the MHC molecule. Furthermore, the capacity of MHC-immunoglobulin–based reagents to identify antigen-specific T-cell responses is limited as compared to MHC exchange tetramers (**Supplementary Fig. 4** online).

The observation that ligands that disintegrate on command could be readily identified for both tested MHC alleles suggests that it will be straightforward to identify 2-nitrophenyl–based conditional ligands for other MHC class I alleles. Conditional ligands can be designed by replacement of amino acids in either a known peptide ligand, or in a predicted high-affinity ligand based on the peptide binding motif for this allele, and for HLA-A2.1 both approaches have been successful. For MHC alleles for which structural information is available, the water-accessibility of side chains may also be used as a criterion to select positions at which the UV light–sensitive building block can be incorporated.

Various types of functional assays for detection of antigen-specific T cells, such as intracellular cytokine staining and cytokine capture, have been developed in the past few years and these assays may be used for high-throughput analysis of T cells that have a given effector function. MHC exchange multimer technology should complement these technologies by allowing high-throughput analysis of T-cell responses, irrespective of the capacity of T cells to produce a given cytokine. Also the combination of the two technologies, in which high-complexity MHC multimer arrays are used to probe T-cell reactivity by monitoring cytokine production, may be particularly useful. On-command cleavage of MHC ligands also seems attractive for the production of clinical-grade MHC reagents for adoptive T-cell therapy[13]. Specifically, the generation of a single large batch of clinical-grade MHC molecules complexed with conditional ligand may allow the straightforward assembly of an MHC reagent desired for clinical use, by performing MHC exchange reactions with the relevant peptide ligands. Such high-grade MHC reagents should be particularly attractive for the isolation of melanoma-specific T cells[29], and for the isolation of defined minor histocompatibility antigen-specific T cells[12]. Finally, in addition to the use of conditional MHC class I ligands for high-throughput diagnostic screening and for adoptive T-cell therapy, we speculate that cleavage of ligands bound to MHC molecules in the crystalline state[30] might be used to obtain the elusive structure of the empty MHC class I molecule.

METHODS

Peptide synthesis and preparation of recombinant MHC. We obtained the UV light–sensitive building block for peptide synthesis (*N*-fluorenylmethyl-oxycarbonyl 3-amino-3-(2-nitro)phenyl-propionic acid) by protection of 3-amino-3-(2-nitro)phenyl-propionic acid (Lancaster) with fluorenylmethyl chloroformate (Sigma-Aldrich) in dioxane–10% aqueous Na_2CO_3 3/2 (vol/vol) according to a published procedure[21]. We synthesized naturally occurring peptides and UV light–sensitive peptide variants by standard Fmoc synthesis. We performed MHC class I refolding reactions as previously described[20], and we purified refolded MHC class I molecules by gel-filtration chromatography on a Phenomenex Biosep SEC S3000 column (Phenomenex) in 20 mM Tris-HCl (pH 7.0), 150 mM NaCl. We stored purified MHC class I complexes at –20 °C in 20 mM Tris-HCl (pH 7.0), 150 mM NaCl and 16% glycerol.

MHC exchange reactions. To produce single or small sets of MHC reagents by MHC exchange, we exposed biotinylated HLA-A2.1–GILGFVFJL, HLA-A2.1–ILAETVAJV or H-2D^b–ASNENJETM complexes (0.5 μM in 20 mM Tris-HCl (pH 7.0), 150 mM NaCl and 0.5 mM dithiothreitol (DTT)) to UV light (366-nm UV lamp; Camag) in the presence of 50 μM of the indicated peptides for 1–2 h on ice. After exchange, we spun samples at 16,000*g* for 5 min, added PE-streptavidin and used the resulting MHC exchange tetramers for T-cell staining without further purification.

To generate the collection of H5N1-A2K^b tetramers, we predicted potential peptide epitopes within four gene segments of influenza A/Vietnam/1194/04 using the SYFPEITHI prediction program[22] and all peptides with a SYFPEITHI score ≥20 were produced by microscale (60 nmol) synthesis (JPT Peptide Technologies, GmbH). Of the 132 potential epitopes, 116 terminated in either a valine, leucine or isoleucine residue and these were synthesized with the naturally occurring C-terminal amino acid. The remaining 16 peptides, terminating in various nonaliphatic amino acids, were all synthesized with a C-terminal isoleucine to facilitate peptide production. We prepared MHC exchange tetramers by performing parallel small-scale exchange reactions on biotinylated A2K^b-GILGFVFJL class I complexes (0.5 μM)[28] with the 132 candidate influenza A/Vietnam/1194/04 epitopes and a set of control peptides (all peptides at 50 μM), by exposure to UV light for 1 h on ice in 20 mM Tris-HCl (pH 7.0), 150 mM NaCl, and 0.5 mM DTT in 96-well tissue-culture V-bottom polystyrene plates (NUNC). Then we spun the samples at 3,300*g* for 5 min, added PE-streptavidin (final concentration, 10 μg/ml) and used the resulting MHC exchange tetramers for T-cell staining without further purification.

Mice and vaccinations. We obtained C57BL/6 mice and mice transgenic for the HLA-A2K^b fusion gene[26] from the animal department of The Netherlands Cancer Institute. All animal experiments were carried out in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of the Netherlands Cancer Institute (DEC).

For live influenza A infections, we intranasally administered 50 μl of HEPES-buffered saline solution (Life Technologies) containing 200 hemagglutinating units influenza A/HKx31 virus to anesthetized mice. For vaccination of HLA-A2 transgenic mice with H5N1 gene segments, we obtained the indicated gene segments from influenza A/Vietnam/1194/04, isolated from an individual in Vietnam and cloned these into pVAX. Groups of four to six mice were vaccinated by DNA tattoo[27] on days 0, 3 and 6 with 20 μg of pVAX expressing either the influenza A/Vietnam/1194/04 M1, hemagglutinin, neuraminidase or NP gene under control of the CMV promoter.

Cells and flow cytometry. For analysis of MHC multimer binding and T-cell responses in mouse samples, we obtained peripheral blood and removed erythrocytes by incubation in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.4)) on ice. Cells were stained with antibody to CD8α (BD Biosciences) and the indicated MHC tetramers for 10–15 min at 15–25 °C.

For analysis of MHC multimer binding and T-cell responses in human samples, we obtained peripheral blood mononuclear cells of healthy volunteers by Ficoll gradient separation. We obtained the MART-1(26–35)–specific CTL clone by repetitive stimulation and cloning of tumor-infiltrating lymphocytes of an individual with melanoma. We stained cells with the indicated MHC tetramers for 5 min at 37 °C. Then we added CD8-specific antibody (BD Biosciences) and incubated cells for 10–15 min at 15–25 °C. Data acquisition and analysis was carried out on a FACSCalibur (Becton Dickinson) using CellQuest software.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We would like to thank V. Cerundolo (Weatherall Institute of Molecular Medicine) for the A2K^b expression construct and F. Lemonnier (Institut Pasteur) for HLA-A2.1-transgenic mice. We would like to thank A. Pfauth and F. van Diepen for flow cytometry assistance, I. Blonk for help in generation of H5N1 DNA vaccines and H. Hilkmann for peptide synthesis. This work was funded by Netherlands Organization for Scientific Research (NWO) Pioneer (to T.S.) and Vidi (to H.O.) grants.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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Chapter 3

Targeting self antigens through allogeneic TCR gene transfer

Moniek A. de Witte*, Miriam Coccoris*, Monika C. Wolkers, Marly D. van den Boom, Elly M. Mesman, Ji-Ying Song, Martin van der Valk, John B.A.G. Haanen and Ton N.M. Schumacher

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Blood 108: 870-877 (2006)

Targeting self-antigens through allogeneic TCR gene transfer

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Adoptive transfer of T-cell receptor (TCR) genes has been proposed as an attractive approach for immunotherapy in cases where the endogenous T-cell repertoire is insufficient. While there are promising data demonstrating the capacity of TCR-modified T cells to react to foreign antigen encounter, the feasibility of targeting tumor-associated self-antigens has not been addressed. Here we demonstrate that T-cell receptor gene transfer allows the induction of defined self-antigen–specific T-cell responses, even when the endogenous T-cell repertoire is nonreactive. Furthermore, we show that adoptive transfer of T-cell receptor genes can be used to induce strong antigen-specific T-cell responsiveness in partially MHC-mismatched hosts without detectable graft versus host disease. These results demonstrate the feasibility of using a collection of "off the shelf" T-cell receptor genes to target defined tumor-associated self-antigens and thereby form a clear incentive to test this immunotherapeutic approach in a clinical setting. (Blood. 2006;108:870-877)

Introduction

Major histocompatibility complex (MHC) molecules present peptides on the cell surface irrespective of whether they are derived from foreign proteins or from self-proteins. Different tissue types within the body each express a unique set of proteins, and peptide epitopes derived from such tissue-specific proteins can in principle be used as tumor-rejection antigens.[1] However, because most of these tumor-associated antigens (TAAs) are nonmutated self-antigens, the T-cell repertoire specific for such antigens is generally small in size and low in avidity. Indeed, both preclinical studies and clinical trials have provided evidence that a lack of T cells with the required reactivity is a major factor limiting T-cell–based immunotherapy. For instance, murine studies have demonstrated that tumor-specific T-cell responses against foreign tumor-associated antigens can readily be induced by vaccination. However, when the same tumor-associated antigen is considered "self" by the available T-cell repertoire, reactivity to these antigens is highly reduced.[2,3] In line with this, replacement of the endogenous T-cell compartment through a combination of allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI) forms an effective treatment strategy for patients with hematologic malignancies such as chronic myeloid leukemia (CML).[4] Importantly, the antileukemic effect of allo-SCT/DLI is dependent on the recognition of minor histocompatibility antigens (MiHAgs) of the recipient as "nonself" by the infused donor lymphocytes, and the development of T-cell responses against such antigens is predictive of remission.[5] The effects of DLI following allo-SCT provide an excellent example of how an endogenous antigen can become foreign by introduction of a novel T-cell compartment and how recognition of endogenous antigens by this exogenous T-cell compartment is associated with remission. The major drawback of this treatment protocol is that the introduced T-cell reactivity against self-antigens is not specifically directed toward defined tissues but is strictly determined by the available MiHAg differences between donor and recipient. As a consequence, allo-SCT/DLI may fail to result in tumor regression in cases where the immunodominant MiHAgs are not expressed on tumor cells. Furthermore, expression of the immunodominant MiHAgs on nonmalignant cell types results in graft versus host disease (GvHD), a common complication of allo-SCT/DLI with severe morbidity and mortality.

To introduce a new T-cell repertoire that is more specifically directed toward tumor cells, it has been suggested to transfer genes encoding TAA-specific T-cell receptors (TCRs) into autologous T cells. As an example, HLA-A2.1–restricted T-cell receptors specific for melanocyte antigens or for MiHAgs may be isolated from patients experiencing tumor remission following adoptive T-cell therapy for melanoma[6,7] or allo-SCT for CML,[5,8] and such TCRs may then be used for the treatment of HLA-A2.1–positive patients that share this disease (hereafter referred as allogeneic TCR gene transfer).

The potential of TCR gene transfer has been demonstrated in a recent series of studies that demonstrated both in vitro[9-11] and in vivo[12,13] that TCR gene transfer suffices to redirect T cells to antigens of choice. However, 2 issues that are essential for the

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Submitted August 8, 2005; accepted March 15, 2006. Prepublished online as *Blood* First Edition Paper, April 6, 2006; DOI 10.1182/blood-2005-08-009357.

Supported by grants from the Netherlands Organization for Scientific Research (NWO Pioneer grant 00-03), the Dutch Cancer Society (NKI 2003-2860), and the EU 6th Framework program (ATTACK).

M.A.d.W. and M.C. designed and performed research, analyzed data, and wrote the paper; M.C.W. designed and performed research; M.D.v.d.B. and E.M.M. performed research; J.-Y.S. analyzed data, M.v.d.V. performed research and analyzed data; J.B.A.G.H. designed research; and T.N.M.S. designed research, analyzed data, and wrote the paper.

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The online version of this article contains a data supplement.

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clinical implementation of TCR gene transfer have not been addressed; first: whether TCR gene transfer can be used to break tolerance toward a defined self-antigen; and second: whether TCR gene transfer is feasible in settings where the TCR recipient and TCR donor are partially MHC mismatched, an essential requirement for the development of collections of tumor-specific TCR genes that can be used for the treatment of larger patient groups.

Here we demonstrate that TCR gene transfer meets these 2 essential requirements for clinical use. First, we show that autologous T cells that are transduced with an allogeneic high-affinity TCR specific for a defined self-antigen can specifically target a tissue or tumor expressing this self-antigen. Second, we demonstrate that TCR-modified T cells do function in a variety of partially MHC-mismatched recipients without detectable off-target reactivity. Based on these data we feel that there is sufficient incentive to analyze the feasibility of inducing defined tumor-specific T-cell responses by TCR gene transfer in clinical trials.

Materials and methods

Mice

C57BL/6 (H-2^b) (B6), Balb.B (H-2^b), and F1 offspring of C57BL/6 and Balb/C (H-2^d), SJL (H-2^s), B10.BR (H-2^k), or FvB (H-2^q) mice were obtained from the Experimental Animal Department of The Netherlands Cancer Institute. RIP-OVA^{hi} mice[14] were kindly provided by Dr C. Kurts (Friedrich-Wilhelms-Universitat, Bonn, Germany). All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute (DEC).

Retroviral constructs and retroviral transduction of T cells

The TCRα and TCRβ fragments of both the F5 and OT-I TCR, separated by an internal ribosomal entry sequence (IRES), were cloned into the pMX retroviral vector[15] to obtain pMX-F5α-IRES-F5β [16] and pMX-OT-Iα-IRES-OT-Iβ constructs. Mouse splenocytes were modified by retroviral transduction as described previously.[12]

Generation of tumor lines expressing ovalbumin and tumor treatment

The C-terminal part of ovalbumin (amino acids [aa] 161 to 385) and the murine CD4 molecule were cloned into the pMX retroviral vector separated by IRES to obtain pMX-OVA-IRES-CD4. B16 cells were transduced with this construct, and the B16-OVA-IRES-CD4 (B16-OVA) cell line was obtained as a single cell clone selected for high CD4 expression. Melanoma cells were washed with HBSS (Gibco) to remove serum components, and 1×10^5 cells were injected in 200 μL HBSS subcutaneously. Tumors were measured with calipers, and mice were killed after tumors reached an average diameter of 12.5 mm.

Flow cytometry

Surface TCR expression was measured 24 hours after transduction by flow cytometry. Cells were stained with FITC- or PE-conjugated anti-TCR Vα2 and anti-TCR Vβ5 monoclonal antibodies (mAbs) (OT-I TCR), anti-TCR Vβ11 mAb and anti-TCR Vβ2, 3, 4, 5.1, 8, 9, and 10b mAb (anti-Vβ-pool) (F5 TCR), or MHC tetramers, in combination with PE- or APC-conjugated anti-CD8α mAb (all mAbs from BD Pharmingen [San Diego, CA] except PE-conjugated anti-CD8α mAb from Caltag [Burlingame, CA]).[17] Propidium iodide (Sigma, St Louis, MO) was used to select for live cells. For the measurement of T-cell responses, peripheral-blood samples were taken at the indicated days after transfer. Following removal of erythrocytes by NH_4Cl treatment, the cells were washed twice with PBS with 0.5% BSA and 0.02% NaN_3 (PBS/BSA). Cells were stained with the relevant antibodies and analyzed by flow cytometry. Intracellular IFN-γ stainings were performed as previously described.[12] Data acquisition and analysis was done on a FACSCalibur (Becton Dickinson, MountainView, CA) with CellQuest software.

Viral infection

For live influenza A infections, anesthetized mice were infected by intranasal administration of 50 μL HBSS (Life Technologies, Grand Island, NY) containing 200 plaque forming units (PFU) of influenza A/WSN/33 (WSN)–OVA(I)[18] virus (hereafter referred to as inflova) or 25 hemagglutinating units (HAU) of influenza A/NT/60/68 virus. For vaccinia infections, 2×10^7 PFU was intraperitoneally injected. Vaccinia recombinant for GFP-$OVA_{257-264}$ was kindly provided by Dr J. Yewdell (National Institutes of Health, Bethesda, MD).[19]

Allogeneic bone marrow transplantation

Transplantation of female B6 bone marrow and splenocytes into lethally irradiated male Balb.B recipients was performed as described previously.[20]

Histopathology

Extensive necropsy was done on F1 mice that received TCR-transduced cells and subsequent viral infection and on control mice that only received a viral infection. Tissues were sampled in buffered formalin (skin, liver, salivary glands, gastrointestinal tract, spleen, pancreas, heart and lungs, urogenital system, secondary sex glands, head, extremities, and spinal cord). Sections were stained with hematoxylin and eosin and examined blindly for indications of autoimmune pathology, with a special emphasis on liver, skin, and intestine. The sections were reviewed with a Zeiss Axioskop2 Plus microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with Plan-Apochroma (×5/0.16, ×10/0.45, ×20/0.60, and ×40/0.95) and Plan-Neofluar (×2.5/0.075) objectives. In addition to the objectives, there was an extra enlargement device included in the body of the microscope. Images were captured with a Zeiss AxioCam HRc digital camera and processed with AxioVision 4 software (both from Carl Zeiss Vision, München, Germany).

Measurement of blood-glucose levels and treatment of diabetes

Blood-glucose levels in RIP-OVA^{hi} mice were monitored by Accu-Check Compact (Roche Diagnostics, Mannheim, Germany) measurement. Mice were considered diabetic when blood-glucose levels were 20 mM or above. For long-time follow-up, diabetic mice were treated with subcutaneous insulin implants according to the manufacturer's protocol (LinShin Canada, Scarborough, ON).

Immunohistochemistry

Immunohistochemistry was carried out on frozen tissue sections. Sections were preincubated with PBS/4% BSA/5% normal goat serum. As primary antibodies, rabbit anti-OVA (Sigma) and rat anti–mouse CD8α (BD PharMingen) were used. Anti-OVA staining was visualized using the Rabbit Envision kit (DAKO, Glostrup, Denmark); anti-CD8 staining was visualized by a 2-step immunoenzymatic procedure. First, biotin-labeled goat-anti–rat immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA) were applied, followed by HRP-labeled avidin-biotin complex (ABC) (DAKO). AEC (Sigma) was used as a substrate chromagen, and slides were counterstained with hematoxylin. Images were processed with the same microscopy device and program as described under "Histopathology."

Results

In vivo function of TCR-transduced T cells in a self-tolerant setting

We first addressed whether TCR gene transfer can be used to break tolerance to a defined tissue-specific self-antigen. To this end, we

examined the feasibility of inducing a β-cell–specific T-cell attack in RIP-OVA^{hi} mice that express the ovalbumin protein in the insulin-producing β cells of the pancreas. To test whether these mice are tolerant toward ovalbumin, RIP-OVA^{hi} mice were infected intranasally with inflova, an influenza A strain recombinant for the MHC class I–restricted epitope of ovalbumin ($OVA_{257-264}$), and peripheral-blood samples were analyzed by MHC-tetramer staining. As an internal control, mice were also analyzed for T-cell responses toward $PR_{366-374}$, the MHC class I–restricted epitope present within the influenza A nucleoprotein of inflova. In none of the RIP-OVA^{hi} mice could $OVA_{257-264}$-specific T cells be detected over background, whereas in control B6 mice the average response was 2.5% of the CD8$^+$ population. This lack of T-cell responsiveness is selective for the self-protein ovalbumin, as the $PR_{366-374}$-specific T-cell responses were of a similar magnitude in both strains (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). To test the absence of OVA-specific CD8$^+$ cells in RIP-OVA^{hi} mice more stringently, splenocytes were isolated 6 weeks after infection for an in vitro restimulation assay with either the $OVA_{257-264}$ or the $PR_{366-374}$ epitope and subsequent intracellular IFNγ staining. In cultures obtained from RIP-OVA^{hi} mice, no CD8$^+$ cells specific for the $OVA_{257-264}$ epitope could be detected, whereas CD8$^+$ cells specific for the $PR_{366-374}$ epitope were present in large numbers (average, 51% of CD8$^+$ cells) (Figure 1). These data show that the $OVA_{257-264}$ epitope is considered "self" in RIP-OVA^{hi} mice and that any residual T-cell reactivity toward this antigen is below background levels.

Having established that the endogenous T-cell repertoire is nonreactive toward the $OVA_{257-264}$ self-epitope, we have used this model to determine whether such self-antigens can be targeted by autologous T cells that are redirected by introduction of a high-affinity TCR that recognizes this antigen. To generate T cells redirected toward ovalbumin, RIP-OVA^{hi}–derived splenocytes were transduced with the OT-I TCR (Vα2$^+$, Vβ5.1$^+$) (Figure S2). After adoptive transfer of 1×10^5 OT-I TCR$^+$ CD8$^+$ cells and subsequent vaccination with inflova, a marked expansion of the Vα2$^+$, Vβ5.1$^+$ population was observed (average, 14%; maximum, 31% of total CD8$^+$ cells). As a control, in RIP-OVA^{hi} mice that received T cells transduced with the F5 TCR that recognizes the MHC class I–restricted epitope of the nucleoprotein of influenza A/NT/60/68 ($NP_{366-374}$), no expansion of the Vα2$^+$, Vβ5.1$^+$ T-cell population could be detected (Figure 2A). Notably, the kinetics and magnitude of the OT-I TCR-modified T-cell response in RIP-OVA^{hi} mice were comparable to T-cell responses observed in B6 mice, indicating that self-tolerance does not affect the capacity of redirected T cells to react to antigen encounter. Furthermore, when RIP-OVA^{hi} mice are rechallenged with antigen 6 weeks later, expansion of the redirected T cells is again comparable with that observed in B6 mice, indicating that redirected T cells maintain the capacity to react to antigen encounter, even in a tolerant host (M.C., unpublished observations, October 2005). If redirected T cells would also be capable of performing effector function in vivo upon encounter of a self-antigen, OT-I TCR-transduced T cells should be able to target the β cells of the pancreas and thereby induce type I diabetes. To examine this, blood-glucose levels were analyzed at multiple time points following infusion of either OT-I or F5 TCR-transduced T cells. After adoptive transfer of OT-I TCR-transduced T cells, more than 80% of the mice developed diabetes within 14 days (blood-glucose levels of 20 mM or above), whereas the control group stayed normoglycemic (Figure 2A). Also, when in vivo activation of OT-I TCR-modified cells was achieved by a different vaccination strategy using a vaccinia strain recombinant for ovalbumin (rVV-OVA), OT-I TCR-transduced T cells proliferated extensively, and β-cell destruction resulting in diabetes was observed in 100% of the mice (Figure 2B). Adoptive transfer of OT-I transduced T cells without vaccination did not result in detectable T-cell responses or the development of diabetes, indicating that endogenous antigen does not induce substantial activation of the infused T cells (data not shown).

To visualize infiltration of OT-I TCR-transduced T cells in the islets of Langerhans, pancreata were harvested 7 days after adoptive transfer of either OT-I or F5 TCR-transduced cells and subsequent inflova infection. CD8$^+$ cells infiltrating the islets of Langerhans were observed only when mice received T cells directed against ovalbumin (Figure 4C-D). Furthermore, the boundary of the β-cell islets of mice that had received OT-I TCR-transduced T cells displayed a jagged appearance, indicating that some β cells had already been killed prior to the clinical onset of diabetes (Figure 4A-B).

If tissue destruction by redirected T cells is restricted to the cell type that expresses the targeted self-antigen, the only clinical effect of adoptive transfer of OT-I TCR-transduced T cells in RIP-OVA^{hi}

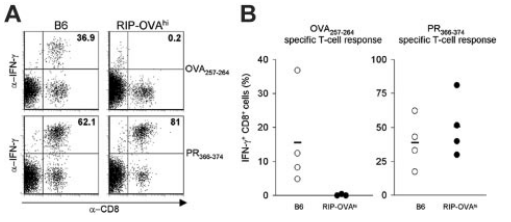

Figure 1. Tolerance in RIP-OVA^{hi} mice. (A) Flow cytometric analysis of in vitro–restimulated splenocytes of inflova-infected B6 (left) or RIP-OVA^{hi} mice (right). An IFNγ assay was performed 14 days after restimulation with 5×10^{-4} μg/mL $OVA_{257-264}$ peptide (top) or $PR_{366-374}$ peptide (bottom). (B) IFNγ production 14 days after restimulation with the $OVA_{257-264}$ peptide (left) or $PR_{366-374}$ peptide (right) of splenocytes from inflova-infected B6 (○) or RIP-OVA^{hi} (●) mice. Each circle represents 1 mouse; bars, averages.

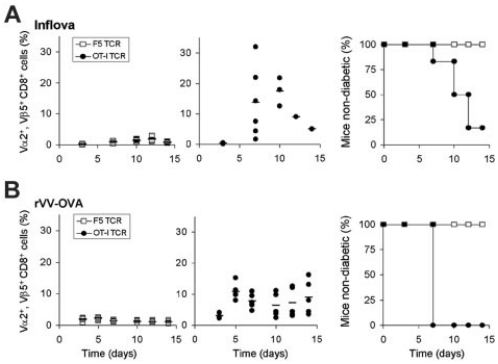

Figure 2. In vivo function of TCR-modified T cells in a self-tolerant setting. (A) Analysis of blood cells and blood-glucose levels of RIP-OVA^{hi} mice that received 1×10^5 F5 (□) or OT-I (●) transduced T cells followed by inflova infection. (B) Analysis of blood cells and blood-glucose levels of RIP-OVA^{hi} mice that received 1×10^6 F5 (□) or OT-I (●) transduced T cells followed by rVV-OVA infection; blood was sampled 3 to 15 days after infection. (Left and middle) Closed circles and open squares represent TCR-transduced T-cell responses in individual mice; bars, averages. (Right) Blood-glucose levels were measured to monitor development of type I diabetes. RIP-OVA^{hi} mice that were infected with inflova were killed upon development of diabetes; RIP-OVA^{hi} mice that were infected with rVV-OVA were treated with insulin implants upon development of diabetes.

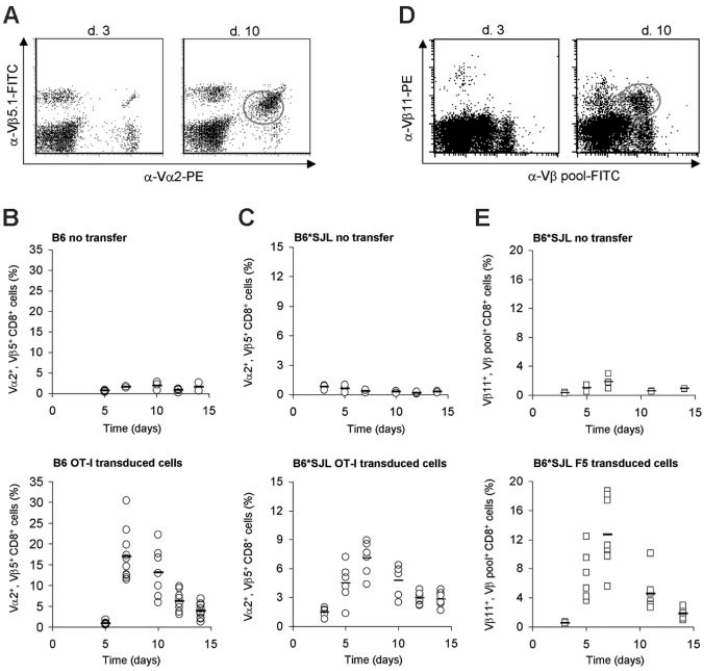

Figure 3. In vivo expansion of TCR-transduced T cells in partially MHC-mismatched recipients. (A) Flow cytometric analysis of blood samples at day 3 (left) or day 10 (right) after infection from B6 mice that received 1×10^5 OT-I TCR-transduced T cells followed by inflova infection. Activated TCR-transduced T cells can be distinguished from the endogenous Vα2/Vβ5$^+$ CD8$^+$ fraction by a lower expression of the Vα2/Vβ5 TCR chains. (B-C) Analysis of blood samples from B6 mice (B) and B6*SJL mice (C) that received no modified T cells (top) or 1×10^5 OT-I TCR-transduced T cells (transduction efficiency in B6*SJL mice, 23% of CD8$^+$ cells) (bottom) followed by inflova infection. Blood was sampled 5 to 14 days after infection and stained as in panel A. Open circles indicate T-cell responses in individual mice; bars, averages. (D) Flow cytometric analysis of blood samples at day 3 (left) or day 10 (right) after infection from B6*SJL mice that received 1×10^5 F5 TCR-transduced T cells (transduction efficiency, 3.5% of CD8$^+$ cells) followed by A/NT/60/68 infection. The percentage of F5 TCR-transduced cells was calculated from the fraction of Vβ11$^+$ cells within the population of Vβ-pool$^+$ cells. Note that a Vβ11^{dull} population that is Vβ-pool negative is present in mice that received F5 TCR-modified T cells. This represents TCR-transduced T cells that express an endogenous Vβ chain for which no antibody was available. (E) Analysis of blood samples from B6*SJL mice that received no modified T cells (top) or 1×10^5 F5 TCR-transduced T cells (bottom) followed by A/NT/60/68 infection. Blood was sampled 5 to 14 days after infection and stained as in panel D. Open squares indicate T-cell responses in individual mice; bars, averages.

mice should be the development of type I diabetes. To address this experimentally, severe diabetes was induced by adoptive transfer of 1×10^6 OT-I TCR-transduced cells and subsequent vaccination with rVV-OVA. This strategy results in a diabetic coma if mice remain untreated. Directly after the onset of diabetes, mice were treated with insulin implants to allow for long-term monitoring. Insulin administration was sufficient to reverse the clinical signs of diabetes such as polydipsia and polyuria. Importantly, following treatment of diabetes, the animals displayed no detectable morbidity during a 3-month follow-up, suggesting that pancreatic β cells are the major or sole target of the infused TCR-modified T cells. Subsequently, pancreata were isolated and analyzed for ovalbumin expression. In mice that received a single adoptive transfer of OT-I cells, the number of islets was decreased approximately 10-fold as compared with control mice (Figure 5). These data demonstrate that a one-time infusion of OT-I TCR-transduced T cells can be used to target a defined tissue-specific self-antigen and that such targeting appears highly selective.

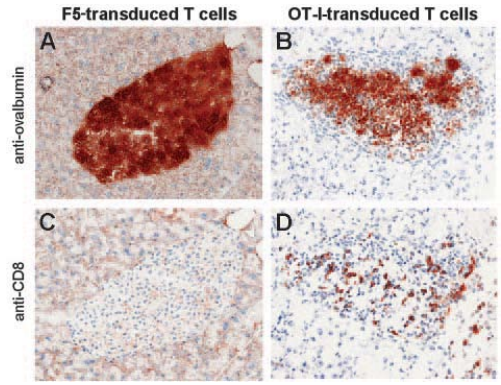

Figure 4. Homing of TCR-transduced T cells to self-antigen–expressing peripheral tissue. RIP-OVA^{hi} mice received 1×10^5 F5 (left) or OT-I (right) transduced T cells followed by inflova infection. Pancreatic islet infiltration by CD8$^+$ cells and islet integrity were determined on day 7. Immunohistochemical analysis for ovalbumin (top) or CD8 (bottom) is shown. Original magnification, ×20.

To address whether TCR-transduced T cells can also target a tumor expressing this antigen in a self-tolerant setting, we generated a variant of the poorly immunogenic B16 melanoma that expresses the C-terminal part of ovalbumin (aa 161 to 407). Importantly, the cells were generated using the murine CD4 molecule as a selection marker to ensure that the marker gene product would not constitute a neoantigen. In line with many prior vaccination studies, when the resulting B16-OVA cells are injected in wild-type B6 mice, for which OVA is a foreign antigen, vaccination suffices to suppress tumor growth. However, the same vaccination is ineffective when RIP-OVA^{hi} are challenged with B16-OVA cells, even when vaccination is given at the day of tumor inoculation (Figure S3). This indicates that the presence or absence of an OVA-specific T-cell repertoire is a critical determinant in this model. To test whether OT-I TCR-transduced T cells could substitute for the absence of a tumor-reactive T-cell repertoire in RIP-OVA^{hi} mice, the mice were challenged with 1×10^5 B16-OVA cells followed by an adoptive transfer of 1×10^6 OT-I transduced cells plus vaccination with rVV-OVA on day 7 after tumor inoculation. Upon inoculation in tumor-bearing mice, OT-I transduced T cells expanded (Figure 6A) and induced severe autoimmune diabetes on days 5 and 6 after infusion (data not shown). Also the tumor was targeted by OT-I TCR-transduced T cells, as shown by a transient regression of the tumor in all mice. This initial

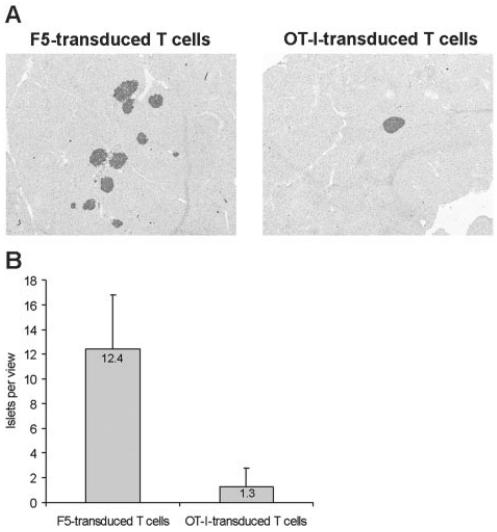

Figure 5. Destruction of ovalbumin-expressing β cells by TCR-transduced T cells. RIP-OVA^{hi} mice received 1×10^6 F5 (left) or OT-I (right) transduced T cells followed by rVV-OVA infection. Mice were treated with insulin implants upon onset of diabetes, and pancreata were harvested 3 months after transfer. Three nonserial sections per mouse were assessed for ovalbumin-positive islands by immunohistochemistry. Per section, the number of islets per view was counted using ×2.5 magnification. Shown are (A) 2 representative sections and (B) the average numbers of islets per view for mice that received F5 or OT-I TCR-transduced T cells. Original magnification, ×5.

regression was followed by a short plateau phase, after which the tumor grew out (Figure 6B). It is noted that in this tumor model, the one-time infusion of OT-I transduced T cells that was used results in a transient response of redirected T cells in peripheral blood that is apparently not maintained by the tumor. Nevertheless, this T-cell response does prolong survival from an average of 21 days in the nontreated group to an average of 38 days in the treated group (Figure 6C).

Safety and feasibility of allogeneic TCR gene transfer in a partially MHC-mismatched setting

In the second part of the study we addressed whether TCR gene transfer is feasible in partially MHC-disparate recipients, in which the introduced TCR will encounter MHC–self-peptide complexes that were not present during thymic selection. In this setting, 2 scenarios could potentially unfold that may impair the feasibility of TCR gene transfer. If T cells transduced with an allogeneic TCR would engage with an allogeneic MHC allele product complexed with a self-antigen with a broad tissue distribution, inactivation of the infused T cells could occur.[21] Alternatively, if TCR-transduced T cells recognize allogeneic MHC molecules complexed with organ-specific self-peptides, the resulting off-target recognition could lead to autoimmune pathology. In addition to these potential problems that are specific for TCR gene transfer in a partially MHC-disparate setting, TCR gene transfer could conceivably lead to off-target autoimmune pathology via 2 other mechanisms (see "Discussion"), and the occurrence of such autoimmune reactivity has only been tested in a small cohort of mice.[12]

To test the feasibility of TCR gene transfer in a large cohort of partially MHC-disparate recipients, we generated offspring of H2^b mice with a number of different non-H2^b strains. Subsequently, peripheral T cells of these mice were used as recipient cells for the F5 TCR and the OT-I TCR. Because both the F5 and OT-I TCR were originally isolated from H-2^b mice, these TCRs are nonreactive with endogenous antigens complexed with the H-2^b class I and class II alleles (H-2K^b, D^b, I-A^b). In line with this, when T cells modified with the F5 TCR[12] or with the OT-I TCR (Figure 3) are adoptively transferred into B6 mice, these cells show a classic proliferative response upon antigen encounter (ie, infection with influenza A strains expressing either the $NP_{366\text{-}374}$ or $OVA_{257\text{-}264}$ epitope).

To determine whether infusion of TCR-modified T cells can also be used to generate antigen-specific T-cell responses in a partially MHC-mismatched setting, splenocytes of F1 offspring of C57BL/6 and Balb/C (H-2^d), SJL (H-2^s), B10.BR (H-2^k), or FvB (H-2^q) mice were transduced with the OT-I TCR, and 1×10^5 OT-I TCR-transduced CD8$^+$ cells were adoptively transferred into syngeneic F1 recipients. Subsequently, mice were intranasally infected with inflova, and T-cell responses were measured in peripheral-blood samples by Vα2, Vβ5.1 staining (Figure 3A). As a control, T-cell responses were followed in F1 mice that had only received inflova virus. In all 4 groups of partially MHC-disparate recipients the OT-I TCR-transduced T cells showed a very marked expansion upon inflova infection with a peak response around day 7 (Figures 3C and S4).

To expand these data to a second T-cell receptor, 1×10^5 F5 TCR-transduced CD8$^+$ cells were adoptively transferred into the various partially MHC-mismatched recipients, followed by an intranasal influenza A/NT/60/68 infection. Because no Vα-specific antibody is available for the F5 TCR and because MHC tetramer

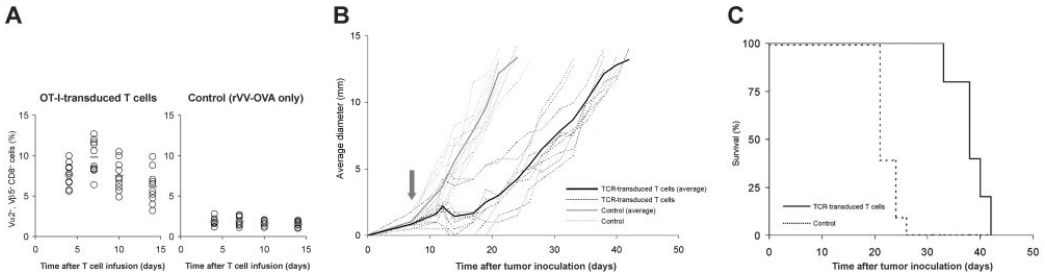

Figure 6. Antitumor effect of TCR-transduced T cells in a self-tolerant setting. RIP-OVA^{hi} mice were challenged with 1×10^5 B16-OVA cells subcutaneously. On day 7, mice received either 1×10^6 OT-I TCR-transduced T cells followed by vaccination with rVV-OVA (left panel in A, black line in B, solid line in C) or vaccination alone (right panel in A, gray line in B, dashed line in C). (A) Blood was sampled and analyzed for the presence of OT-I transduced T cells on day 4 to 14 after adoptive transfer. Each circle represents one mouse; bars, average immune responses. (B) Tumor growth was measured 3 times a week starting at day 7 after inoculation. Dashed lines represent growth curves in individual mice; solid lines, average growth curves; arrow, the start of treatment. (C) Survival curve: Mice were killed when the average tumor diameter exceeded 12.5 mm.

staining does not allow one to distinguish between endogenous and exogenous $NP_{366-374}$-specific T cells, we developed an alternative procedure for tracing TCR-modified T cells ex vivo. Rearrangement of TCRβ genes is subject to allelic exclusion and, as a consequence, conventional αβ T cells only express a single TCRβ subunit.[22,23] In contrast, TCR gene–modified T cells express both the endogenous TCR and the newly introduced TCR and may therefore be detected by screening for such dual Vβ expression (Figure 3D). As was the case for the OT-I TCR-modified T cells, F5 TCR-transduced T cells showed a very marked expansion upon viral infection in all groups of partially MHC-mismatched recipients tested. Consistent with the notion that these $V\beta11^{dull}V\beta\text{-pool}^{+}$ cells represent F5 TCR-modified T cells, no population of $V\beta11^{dull}V\beta\text{-pool}^{+}$ cells was detectable in control groups (Figures 3E and S5).

To examine the occurrence of possible TCR gene transfer–associated autoimmunity in this large group of animals, all partially MHC-mismatched mice that received TCR-modified T cells were clinically observed for at least 6 weeks after transfer. Mortality induced by transfer of TCR-modified cells in MHC-disparate recipients was 0% (0 of 66 mice), and none of the mice developed clinically manifest autoimmune disease. To screen for autoimmune pathology in more detail, all mice were killed to perform an extensive pathologic examination. Special attention was given to gut, liver, and skin, the classic target organs of GvHD (Tables S1-S2).[24] In all groups, no signs of pathology were observed in intestine and skin. In liver tissue, a mild inflammatory process could be observed in most animals. These lesions displayed a random distribution rather than the periportal localization that is a hallmark of liver GvHD. More importantly, the observed liver pathology was found not only in mice that received TCR-transduced T cells (OT-I, 90%; F5, 76%) but also in control groups ("only inflova," 71%; "only influenza A/NT/60/68," 83%), indicating that this pathology is unlikely to be a consequence of the adoptive transfer of TCR-transduced T cells (Figure 7A-B). In addition, in many of the mice that were infected with inflova, pneumonia-related lesions were detected. Again, these lesions were found irrespective of transfer of OT-I TCR-transduced T cells, strongly suggesting that this pathology is virus induced (Table S1). Apart from these 2 histopathologic observations detected irrespective of TCR gene transfer, no consistent pattern of autoimmune pathology was observed (Tables S1-S2). To be able to compare these data to a situation in which GvHD is known to occur, we transplanted MHC-matched bone marrow from B6 into Balb.B mice, where minor histocompatibility differences between the 2 strains are known to result in acute liver and gastrointestinal (GI) tract GvHD.[25] Balb.B recipients of B6 bone marrow developed clinical GvHD on days 18 to 24, upon which they were killed. Histopathologic analysis of these mice revealed severe colitis (100% of mice) and liver pathology with periportal lymphoid infiltrates (89% of mice) characteristic of GvHD (Figure 7C; Table S3).

To test for possible chronic GvHD in mice that received TCR-modified T cells, we looked for signs of autoimmunity in F1 offspring of B6 and Balb/C mice 8 months after transfer of OT-I TCR-transduced T cells. Also during this long-term follow-up, the mice displayed no clinical autoimmunity, and pathologic examination again did not reveal any sign of autoimmunity above that observed in control mice. Collectively, these data demonstrate that for 2 different TCRs in 4 different groups of partially MHC-mismatched recipients tested, adoptive transfer of TCR-transduced T cells does not lead to detectable off-target autoimmunity.

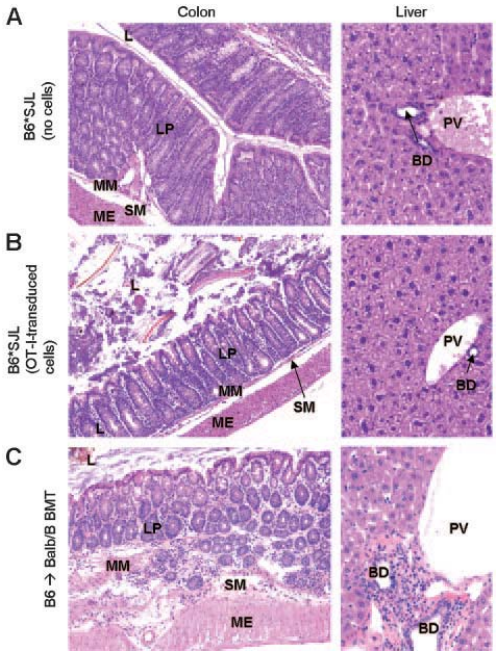

Figure 7. TCR-transduced T cells do not induce graft versus host pathology in partially MHC-mismatched recipients. Colon and liver sections were obtained either 6 weeks after viral infection (A-B) or at the onset of clinical GvHD (C). L indicates lumen; LP, lamina propria; MM, muscularis mucosae; SM, submucosa; ME, muscularis externa; BD, bile duct; PV, portal vein. Lymphocytic infiltrates are present throughout the intestinal wall and in the periportal area of Balb.B recipients (C), whereas intestine and periportal area of partially MHC-mismatched recipients are unaffected (A-B). Original magnification, ×16 (A-B, left panels); ×32 (A-B, right panels); ×125 (C, left panel); and ×20 (C, right panel).

Discussion

Most human tumors do not have a viral etiology, and the shared antigens that are available for T-cell attack are therefore largely restricted to nonmutated self-antigens. As a consequence, the success of T-cell–based immunotherapy relies in large part on the ability to generate tumor-specific T cells that can efficiently target defined self-antigens.

In a murine model expressing a β-cell–specific self-antigen, we have demonstrated that T cells transduced with a TCR recognizing this self-antigen expand upon vaccination and target both pancreatic cells and tumor cells expressing this self-antigen. These data provide the first in vivo evidence that TCR gene transfer can be used to generate a T-cell compartment specific for self-antigens and thereby overcome self-tolerance. It is noted that the effect of single-dose infusion of TCR-transduced T cells left approximately 10% of β-cell islets intact and also is insufficient to result in complete tumor regression. The fact that the response of the TCR-transduced T cells is transient and apparently not maintained by either β-cell or tumor-cell–derived antigen is likely to be important in this respect, and an obvious next step will be the analysis of the effect of repetitive infusion/vaccination.[26,27] In

addition, it seems useful to test the value of additional treatments that are currently used in clinical trials such as lymphodepletion and anti-CTLA4 treatment for a possible potentiating effect. Such protocols may first be evaluated in the type of self-tolerant models used in the present study but should ultimately be validated in murine models for sporadic tumor development, where tumor-induced immune suppression may form an obstacle.[28]

In this study we circumvented self-tolerance by introduction of an ovalbumin-specific TCR that was originally obtained from a mouse for which OVA was not a self-antigen and that was therefore of high affinity. Although we did not compare these data with the adoptive transfer of a low-affinity OVA-specific TCR, prior data on the analysis of the antitumor effects of low-avidity self-specific T cells make it likely that the use of a high-affinity T-cell receptor is essential (reviewed by de Visser et al[29]). Analogous to the development of platforms for the isolation of high-affinity antibodies against human antigens, several strategies to obtain high-affinity TCRs specific for human tumor-associated self-antigens have been described in recent years (reviewed by Coccoris et al[30]), and a comparison of the specificity and activity of TCRs obtained by the methods will be an important goal for the coming period.

In the second part of this study, we have demonstrated—for 2 TCRs—that TCR transfer into T cells of a large cohort of partially MHC-mismatched recipients results in redirected T cells that function in vivo and that this experimental therapy is not associated with detectable autoimmunity. While TCR-gene modified T cells have displayed the intended MHC and antigen specificity in in vitro assays,[31] infusion of TCR-transduced T cells could—at least in theory—induce "off-target" autoimmunity via 3 different mechanisms.[32] Specifically, introduction of an exogenous TCR will not only result in expression of the introduced TCR, but the introduced T-cell receptor chains can also form mixed heterodimers with endogenous T-cell receptor α and β chains, and such mixed TCRs may potentially be reactive toward self-peptides. Secondly, the TCR may be introduced into ignorant self-reactive T cells.[33] If such T cells become activated via the introduced TCR, this will result in an expanded population of activated autoreactive T cells with an increased ability to infiltrate peripheral tissues. If either of these 2 mechanisms would result in autoimmune pathology with appreciable frequency, such pathology would be expected to occur irrespective of possible MHC disparities between TCR donor and recipient. The third mechanism via which TCR-transduced T cells could induce off-target autoimmunity is when the introduced TCR recognizes MHC molecules in the recipient that were absent in the original TCR donor. In this study we have shown the safety of TCR gene transfer in a group of more than 60 partially MHC-mismatched recipient mice, and these results reveal 2 things. First, these data suggest that activation of ignorant self-specific T cells or the formation of mixed heterodimers does not pose a serious risk factor for the development of autoimmune disease upon TCR gene transfer. Second, these data demonstrate that MHC mismatches between TCR donor and recipient can be compatible with effective and safe allogeneic TCR gene transfer. In line with this, an absence of detectable side effects has also been observed in clinical trials in which EBV- and CMV-specific T-cell lines were used for the treatment or prophylaxis of posttransplantation viral infections, although in this case in general under conditions of immunosuppression[34] While encouraging, it is important to note that both sets of data should not be taken as evidence that TCR transfer will never be complicated by MHC mismatches between TCR donor and recipient, because the propensity for reactivity with allogeneic MHC varies between different TCRs. Rather, the data should be taken to indicate that it is feasible to identify TCRs that are safe and effective in a large number of partially MHC-mismatched recipients. As a consequence, it should be possible to generate collections of TCR genes that can be used to induce desired TAA-specific T-cell responses in clinical trials. Should clinical trials nevertheless reveal undesired alloreactivity, it may be possible to limit such toxicity by inclusion of a suicide switch (reviewed by Straathof et al[35]).

Prior data have shown the capacity of TCR gene-modified T cells to react to foreign antigen encounter in immunocompetent mice. The current data expand these studies by demonstrating the feasibility of targeting a defined self-antigen by TCR gene transfer and by demonstrating the lack of toxicity of TCR gene transfer in the clinically relevant setting of a partial MHC disparity. Collectively these studies provide sufficient support to test the safety and efficacy of TCR gene therapy in clinical trials. Such trials will be essential to define under which conditions adoptive immunotherapy with T-cell receptors can be of clinical value and should provide further leads for optimization in preclinical models.

Acknowledgments

We thank M. Toebes for technical support, C. Kurts for the RIP-OVA^{hi} mice, D. Topham for the influenza A/WSN/33 (WSN)–OVA(I) virus, and J. Yewdell for vaccinia virus recombinant for GFP-OVA$_{257-264}$.

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Supplementary tables and figures chapter 3

Supplementary Table I. Pathology after OT-I TCR modified T cell transfer in partially MHC-mismatched recipients

			PATHOLOGY		
TCR	**GROUP**	**ORGAN***	**none**	**mild**	**severe**
OT-I	B6*SJL (n=8)				
		SPLEEN	5/8	3/8 B cell activation	0/8
		G.I. TRACT	7/8	1/8 mild acute gall bladder inf.	0/8
		LUNG	4/8	4/8 pneumonia lesions	0/8
		SKIN	5/8	3/8 mild dermatitis foci	0/8
		LIVER	2/8	6/8 mild inflammation	0/8
-	B6*SJL (n=3)				
		SPLEEN	2/3	1/3 B cell stimulation	0/3
		G.I. TRACT	3/3	0/3	0/3
		LUNG	2/3	1/3 mild inflammation	0/3
		SKIN	38414	0/3	0/3
		LIVER	38355	2/3 mild inflammation	0/3
OT-I	B6*FVB (n=9)				
		SPLEEN	4/9	5/9 B cell stimulation	0/9
		LUNG	2/9	7/9 pneumonia lesions	0/9
		LIVER	2/9	7/9 mild inflammation	0/9
-	B6*FVB (n=3)				
		SPLEEN	2/3	1/3 large B cell stimulation	0/3
		LUNG	2/3	1/3 pneumonia lesions	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
OT-I	B6*B10BR (n=7)				
		SPLEEN	6/7	1/7 B cell activation	0/7
		G.I. TRACT	6/7	1/7 eusophagal mucosa inflamm.	0/7
		LUNG	4/7	3/7 pneumonia lesions	0/7
		SKIN	6/7	1/7 superficial dermatitis	0/7
		LIVER	0/7	7/7 mild inflammation	0/7
		SAL.GLAND	6/7	1/7 mild parotis inflammation	0/7
-	B6*B10BR (n=3)				
		SPLEEN	3/3	0/3	0/3
		G.I. TRACT	2/3	1/3 inflamm. stomach mucosa	0/3
		LUNG	2/3	1/3 pneumonia lesions	0/3
		SKIN	3/3	0/3	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
		SAL.GLAND	2/3	1/3 mild parotis inflamm.	0/3
OT-I	B6*BALB/c (n=9)				
		G.I. TRACT	7/9	1/9 gastritis, 1/9 mild inflamm.	0/9
		LUNG	6/9	3/9 pneumonia lesions	0/9
		LIVER	0/9	9/9 mild inflammation	0/9
		SAL.GLAND	8/9	1/9 mild inflammation	0/9
-	B6*BALB/c (n=3)				
		G.I. TRACT	2/3	1/3 mild inflamm. gastric mucosa	0/3
		LUNG	2/3	1/3 pneumonia lesions	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
		SAL. GLAND	3/3	0/3	0/3

*Only organs with histopathologic abnormalities in either test or control group are shown

Supplementary Table II. Pathology after F5 TCR modified T cell transfer in partially MHC-mismatched recipients.

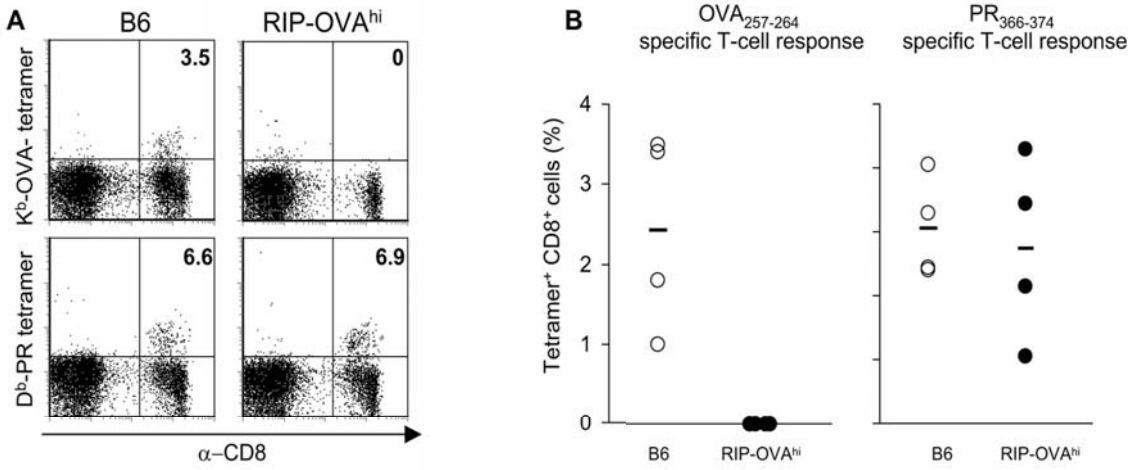
			PATHOLOGY		
TCR	**GROUP**	**ORGAN***	**none**	**mild**	**severe**
F5	B6*SJL (n=8)				
		SPLEEN	0/8	8/8 B cell stimulation	0/8
		G.I. TRACT	8/8	0/8	0/8
		SKIN	7/8	1/8 superficial inflamm.	0/8
		SAL.GLAND	7/8	1/8 periductal infiltrate	0/8
		LIVER	3/8	5/8 mild inflammation	0/8
		JOINTS	8/8	0/8	0/8
-	B6*SJL (n=3)				
		SPLEEN	0/3	3/3 B cell stimulation	0/3
		G.I. TRACT	2/3	1/3 slight inflamm. coecum	0/3
		SKIN	3/3	0/3	0/3
		SAL.GLAND	3/3	0/3	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
		JOINTS	2/3	1/3 very mild arthritis	0/3
F5	B6*FVB (n=8)				
		SPLEEN	1/8	7/8 B cell stimulation	0/8
		LIVER	3/8	5/8 mild inflammation	0/8
-	B6*FVB (n=3)				
		SPLEEN	3/3	0/3	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
F5	B6*B10BR (n=8)				
		SPLEEN	6/8	2/8 B cell stimulation	0/8
		LUNG	7/8	1/8 slight focal lesion	0/8
		LIVER	2/8	6/8 mild inflammation	0/8
		SAL.GLAND	5/8	3/8 mild inflammation	0/8
-	B6*B10BR (n=3)				
		SPLEEN	2/3	1/3 B cell stimulation	0/3
		LUNG	2/3	1/3 infiltrates/vasculitis	0/3
		LIVER	0/3	3/3 mild inflammation	0/3
		SAL.GLAND	1/3	2/3 mild inflammation	0/3
F5	B6*BALB/c (n=8)				
		G.I. TRACT	7/8	1/8 mild gastritis	0/8
		LIVER	0/8	8/8 mild inflammation	0/8
-	B6*BALB/c (n=3)				
		G.I. TRACT	3/3	0/3	0/3
		LIVER	0/3	3/3 mild inflammation	0/3

*Only organs with histopathologic abnormalities in either test or control group are shown

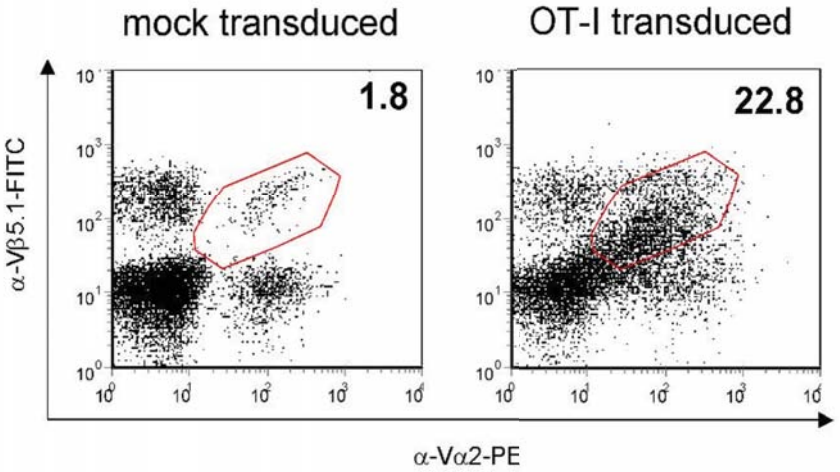
Supplementary Table III. Pathology after B6 BMT into Balb/B recipients.

			PATHOLOGY	
BMT	**GROUP**	**ORGAN***	**none/mild**	**severe**
B6	Balb.B (n=9)			
		SPLEEN	0/9	9/9 hematopoietic activity *
		GI TRACT	0/9	9/9 enteritis with lymphocytic infiltrates and (3/9) necrotic lesions
		SKIN	9/9	0/9
		LIVER	1/9	8/9 lymphocytic inf. in periportal areas and (2/9) bile duct thickening
		TESTIS	0/9	9/9 atrophy *
		BONE MAR	0/9	9/9 degeneration *

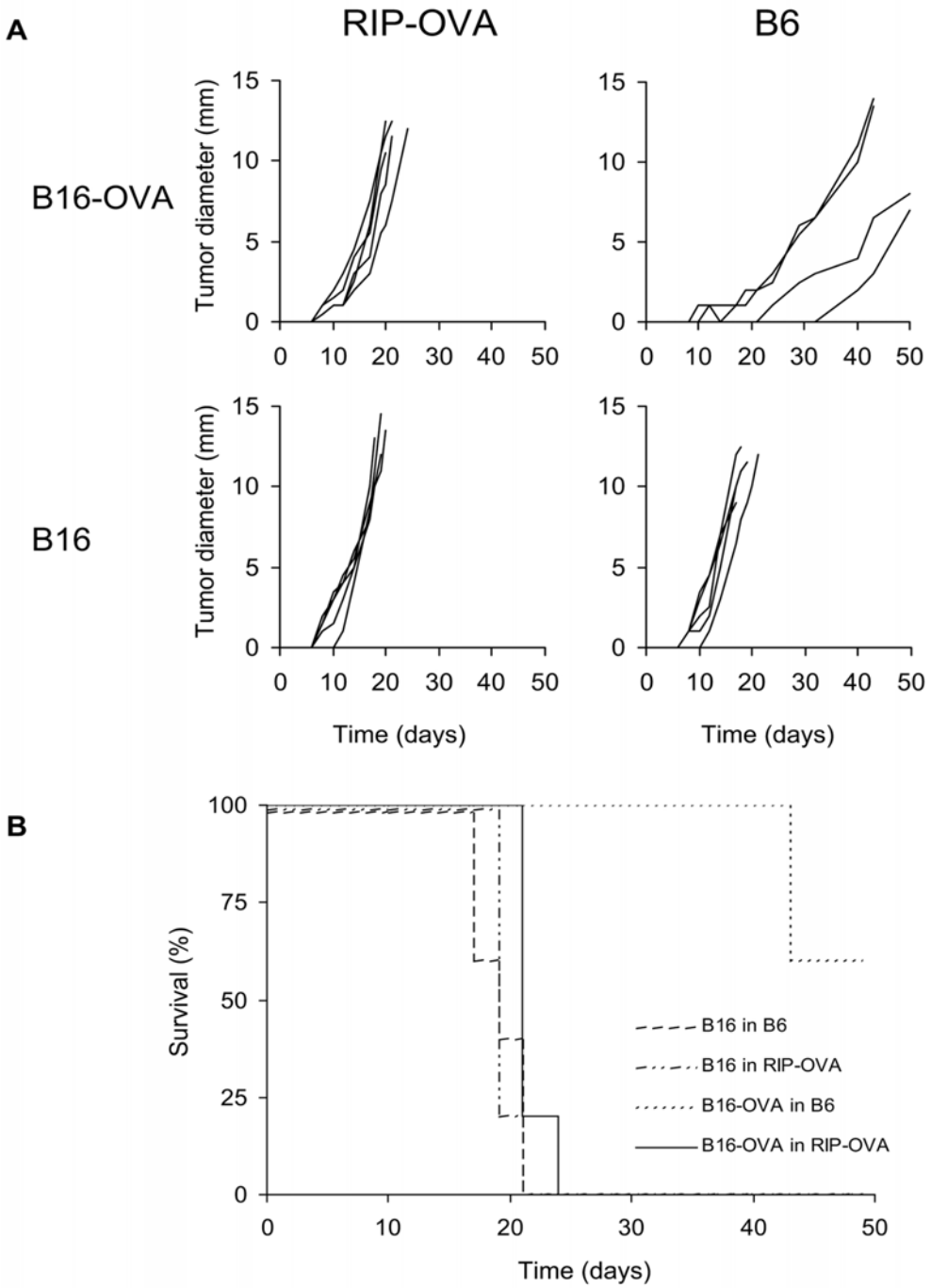
*Post irradiation pathology



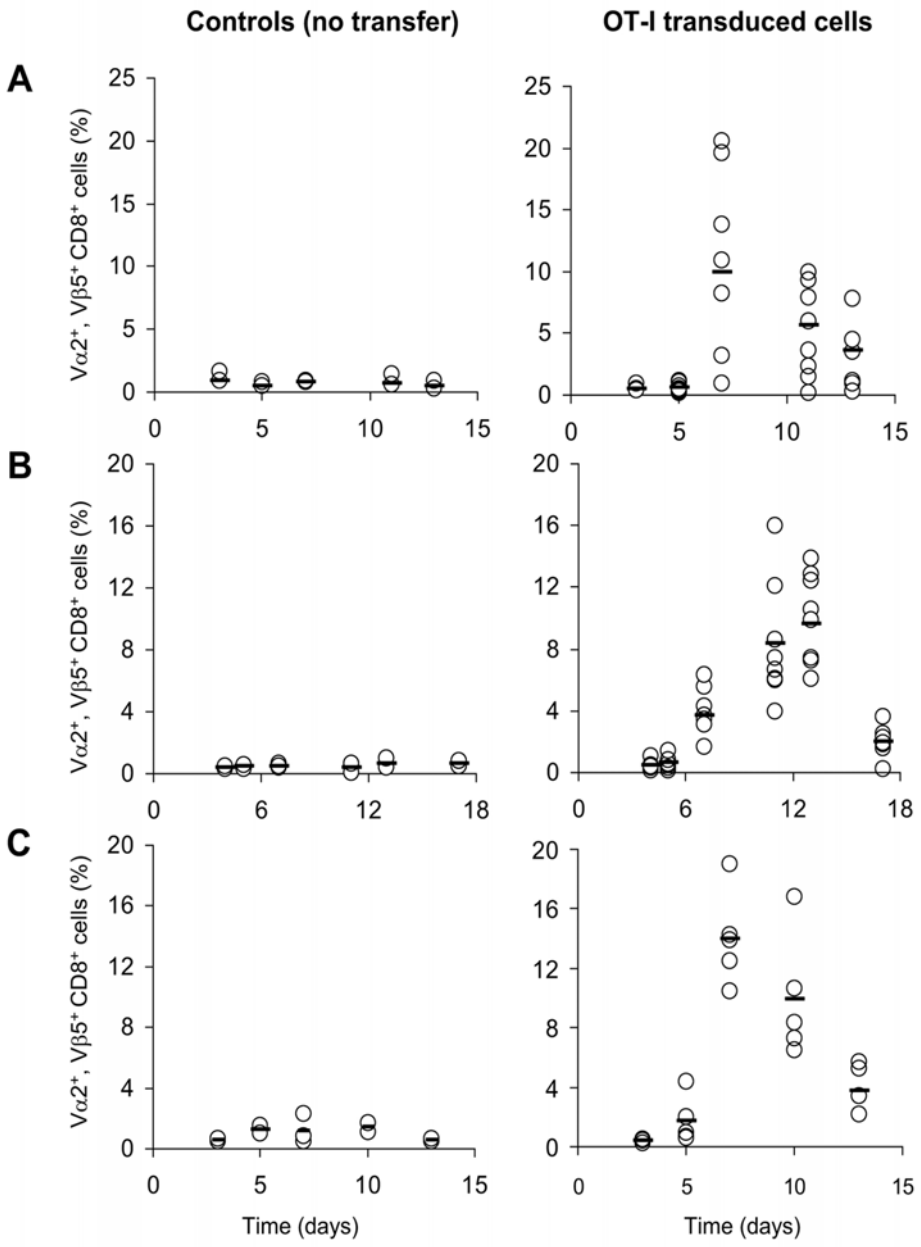
Supplementary Figure 1. Tolerance in RIP-OVA^{hi} mice.
(A) Flow cytometric analysis of blood samples of influenza-infected B6 (left panels) or RIP-OVA^{hi} (right panels) at day post infection. (B) Immune responses towards the $OVA_{257-264}$ epitope (left panel) or $PR_{366-374}$ epitope (right panel) at day 8 post influenza infection in B6 mice (open circles) or RIP-OVA^{hi} mice (closed circles). Circles represent individual mice; bars indicate averages.



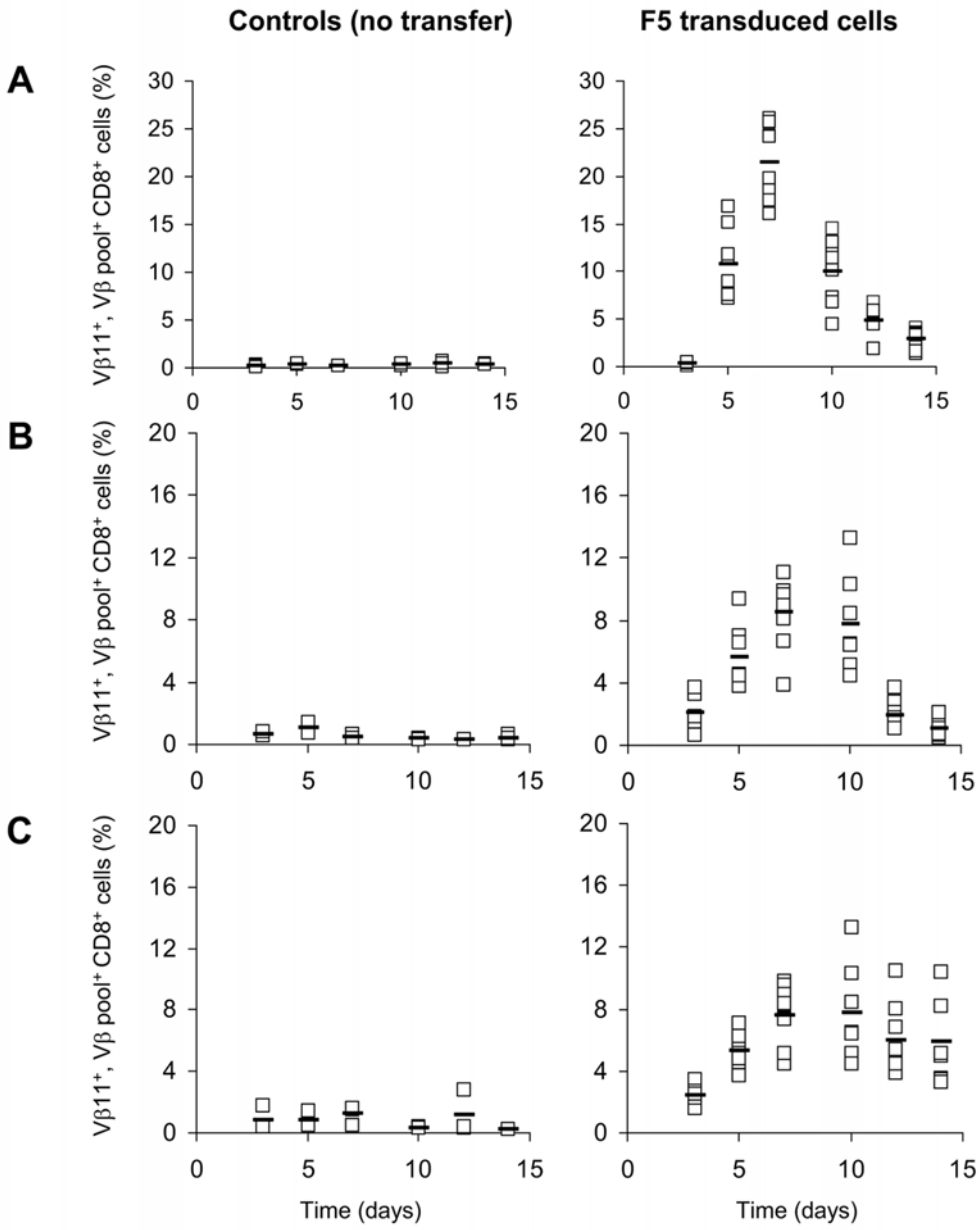
Supplementary figure 2. Analysis of OT-I TCR modified T cells prior to adoptive transfer.
Flow cytometric analysis of mock (left) or OT-I (right) transduced T cells prior to adoptive transfer. The number in the right upper corner of each dot plot reflects the percentage of V2, V5 double-positive cells within the CD8+ population. OT-I transduction efficiency in this experiment was 21%, and to transfer 1×10^5 OT-I+ CD8+ T cells, 4.8×10^5 T cells were infused. Within the different experiments in the RIP-OVAhi mice, OT-I transduction efficiencies varied between 15% and 50%.



Supplementary Figure 3. No immunogenicity of B16-OVA tumors in RIP-OVA^{hi} mice. (A) RIP-OVA^{hi} mice (left) and B6 mice (right) were challenged with 1 × 105 B16-OVA (top) or B16 cells (bottom) subcutaneously, followed by vaccination with rVV-OVA at the same day. Tumor growth was measured 3 times a week, starting at day 4 after inoculation.Lines represent growth curves in individual mice.(B) Survival curve: Mice were killed when the average tumor diameter exceeded 12.5 mm.



Supplementary Figure 4. Ag-driven expansion of OT-I TCR modified T cells in partially MHC-mismatched recipients. Analysis of blood cells from B6xB10BR (A), B6xBalb/c (B) and B6xFVB (C) mice that received no modified T cells (left panels) or $1x10^5$ OT-I TCR modified T cells (right panels) followed by inflova infection. Blood was sampeled 3-14 days after infection and stained with Vβ5.1, Vα2 and CD8α mAbs. Circles represent individual mice; bars indicate averages.



Supplementary Figure 5. Ag-driven expansion of F5-TCR modified T cells in partially MHC-mismatched recipients. Analysis of blood cells from B6xB10.BR (A), B6xBalb/c (B) and B6xFVB (C) mice that received no modified T cells (left panels) or $1x10^5$ F5 TCR modified T cells (right panels) followed by influenza A/NT/60/68 infection. Blood was sampeled 3-14 days after infection and stained with Vβ11, Vβpool and CD8α mAbs. Circles represent individual mice; bars indicate averages.

Chapter 4

Long term *in vivo* functionality of TCR transduced T cells

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(* these authors contributed equally to this work)

Journal of Immunology 180: 6536-6543 (2008)

Long-Term Functionality of TCR-Transduced T Cells In Vivo[1]

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To broaden the applicability of adoptive T cell therapy to cancer types for which tumor-specific T cells cannot routinely be isolated, an effort has been made to develop the transfer of tumor-specific TCR genes into autologous T cells as a novel immunotherapeutic approach. Although such TCR-modified T cells have been shown to react to Ag encounter and can be used to break tolerance to defined self-Ags, the persistence and capacity for renewed expansion of TCR-modified T cells has not been analyzed. To establish whether TCR-transduced T cells can provide recipients with long-term Ag-specific immune protection, we analyzed long-term function of TCR transduced T cells in mouse model systems. We demonstrate that polyclonal populations of T cells transduced with a class I restricted OVA-specific TCR are able to persist in vivo and respond upon re-encounter of cognate Ag as assessed by both proliferation and cytolytic capacity. These experiments indicate that TCR gene transfer can be used to generate long-term Ag-specific T cell responses and provide a useful model system to assess the factors that can promote high-level persistence of TCR-modified T cells. *The Journal of Immunology,* **2008, 180: 6536–6543.**

Adoptive cell transfer (ACT)[6] of tumor-infiltrating lymphocytes (TIL) in combination with nonmyeloablative chemotherapy has been the major success of immunotherapy for melanoma in recent years (1, 2). Importantly, in a proportion of patients, substantial numbers of the adoptively transferred cells remain detectable for periods of months and retain antitumor reactivity. Furthermore, a strong persistence of the adoptively transferred cells in the months following transfer has been shown to correlate with tumor regression (3). These data suggest that T cell-based therapies should aim for conditions where therapy-induced reactivity is not transient, but persists for prolonged periods of time.

Although the clinical results obtained with ACT for melanoma have been impressive, TIL cultures that can be used for infusion cannot be obtained for all melanoma patients. More importantly, for essentially all other human cancers, the routine generation of highly tumor-reactive TIL cultures has not been successful. With the aim to develop protocols for adoptive therapy that do not rely on the availability of TIL, it has been proposed to provide autologous T cells with tumor cell-specificity, by genetic introduction of a tumor-specific TCR (4, 5). The in vivo function of TCR modified T cells has been studied extensively in mouse models. Data obtained in these studies indicate that both cytotoxic (6) and helper T cell compartments (7, 8) with a defined Ag reactivity can be generated and these cells can expand to high numbers upon in vivo Ag encounter (6, 9). Furthermore, infusion of TCR-modified T cells can be used to circumvent self-tolerance to defined self-Ags (9), including Ags expressed in spontaneous tumor models (M. de Witte, G. Bendle, M. van den Boom, M. Coccoris, T. Schell, S. Tevethia, E. Mesman, J. Song, and T. Schumacher, submitted for publication). Finally, a recent phase I clinical trial has demonstrated that TCR gene transfer is feasible in a clinical setting (10). In this trial, clinical responses were relatively rare and correlated with persistence of the TCR modified T cells. However, whether the limited persistence seen in the majority of patients is an intrinsic limitation of TCR modified T cells is unknown.

In the adoptive cell transfer protocol developed by the Rosenberg group that is based on the use of ex vivo expanded tumor-infiltrating lymphocytes, in vivo persistence of the infused cells correlates with telomere length and capacity for CD27 expression (11, 12). These data suggest that in this protocol, in vivo T cell persistence is primarily controlled by the proliferative capacity of the infused cell population. Based on these results, and on analogous data in mouse models (13), the development of culture conditions that yield tumor-specific T cells with high proliferative potential has become a major goal in ACT.

Importantly, besides the proliferative potential of the infused cells, additional factors may influence T cell persistence in case of TCR gene-modified T cells. The retroviral introduction of a tumor-specific TCR may lead to the recognition of the adoptively transferred cells by the host immune system. Such recognition could be based on the MHC-restricted presentation of epitopes in cryptic open reading frames in the viral vector used for gene modification. Alternatively, the nongermline encoded CDR3 segments of the introduced TCR may be recognized as foreign, either by host T cells or by host Abs (14). In particular, this second potential cause of immunogenicity would be problematic as the introduction of nongermline encoded TCR sequences is inherent to the strategy. In addition to the potential immunogenicity of the infused cells, the ex vivo retroviral modification of T cells may affect their capacity to form T cell memory. Before retroviral transduction, T cells are activated nonspecifically by either anti-CD3 Abs or lectins to allow retroviral gene delivery. However, the costimulatory signals

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Received for publication June 11, 2007. Accepted for publication March 10, 2008.

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[1] This research was supported by the European Union 6th Framework program (ATTACK).

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[6] Abbreviations used in this paper: ACT, adoptive cell transfer; TIL, tumor-infiltrating lymphocyte; WSN-OVA, A/WSN/33-OVA virus; rVV-OVA, recombinant vaccinia virus encoding OVA.

via accessory molecules such as CD28, CD27, and 4-1BB that naive T cells receive from APCs during in vivo T cell activation are likely lacking during the in vitro activation steps used in retroviral transduction procedures. Furthermore, CD4 T cell help has been reported to be important for the development of a functional CD8 T cell memory cell pool, possibly through the induction of IL-2 expression (15–18), and it is unclear to what extent the in vitro T cell activation conditions used for retroviral T cell modification can provide these signals. Finally, arguably the greatest value of infusion of TCR gene-modified T cells lies in the treatment of human malignant disease in cases where an endogenous tumor-specific T cell repertoire is lacking due to immunological self-tolerance. In such cases, the function of infused TCR-modified T cells specific for self-Ags may be hampered by regulatory T cells, or TCR-modified T cells may become tolerized during Ag encounter in vivo.

In view of the apparent value of long-term persistence of the tumor-specific T cell response induced by ACT, in this study, we have assessed the long-term functionality of TCR-modified T cells in both nontolerant and self-tolerant settings.

Materials and Methods

Mice

C57BL/6 (B6) mice, C57BL/6LY5.1/5.2 (B6LY5.1/5.2) F_1 mice, OT-I mice (20), and RIP-OVA^{high} mice (21) were bred in the Experimental Animal Department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). All experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute.

Virus infections

For live virus infections, anesthetized mice were infected by intranasal administration of 50 μl HBBS (Life Technologies) containing 200 PFU of A/WSN/33-OVA virus (WSN-OVA, Inflova) (22). For infections with vaccinia recombinant for GFP-OVA$_{257–264}$ (rVV-OVA) (23) mice were injected i.p. with 2×10^9 PFU.

Isolation and retroviral transduction of T cells

Splenocytes were harvested from donor mice and single-cell suspensions were prepared by transferring cell suspensions through a nylon filter (NPBI, Emmer-Compascuum). Erythrocytes were lysed by NH_4Cl treatment and the remaining cells were washed. For retroviral transductions, total mouse splenocytes were cultured in 24-well plates (3×10^6 cells per well) for 48 h in RPMI 1640 medium (Life Technologies BV) supplemented with 8% FCS (BioWhittaker), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Roche Diagnostics) in the presence of Con A (2 μg/ml) (Calbiochem) and IL-7 (IL-7, 1 ng/ml) (Santa Cruz Biotechnology) before transduction.

Retroviral supernatants were obtained by transfection of pMX-OT-Iα-IRES-OT-Iβ TCR DNA (24) or the pMX-barcode library (K. Schepers, E. Swart, J. van Heijst, C. Gerlach, M. Castrucci, D. Sie, M. Heimerikx, A. Velds, R. Kerkhoven, and R. Arens, submitted for publication) and pCLeco (Imgenex) into Phoenix-E packaging cells with the use of FuGENE 6 transfection reagent (Roche Diagnostics). Non-tissue-culture-treated Falcon 24-well plates (BD Biosciences) were coated with 0.5 ml of 30 μg/ml recombinant human fibronectin fragment CH-296 (RetroNectin, Takara) at room temperature for 2 h. The CH-296 solution was removed and replaced with 1 ml of 2% BSA in PBS for 30 min at room temperature. The target cells were plated on the RetroNectin-coated plates (3×10^6 cells per well) in 0.5 ml of retroviral supernatant. Plates were subsequently centrifuged for 90 min at 2,000 rpm, after which the cells were cultured at 37°C. After 24 h, cells were harvested and dead cells were removed by Ficoll-Paque (Merck) density gradient centrifuging. Transgene expression was determined by flow cytometry. Cells were washed with HBSS twice. Finally, cells were resuspended in HBSS and injected i.v. in mice.

Analysis of T cell responses

Surface TCR expression was measured 24 h after transduction by flow cytometry. Cells were stained with PE-conjugated anti-TCR Vα2 mAb, FITC-conjugated anti-TCR Vβ5.1/5.2 mAb, and allophycocyanin-conjugated anti-CD8α mAb (all mAbs from BD Pharmingen). To determine TCR-transduced T cell responses in peripheral blood, samples were taken at the indicated days post transfer. Following removal of erythrocytes by NH_4Cl treatment, the cells were washed twice with PBS with 0.5% BSA and 0.02% NaN_3. Cells were stained with the following Abs: allophycocyanin-conjugated anti-LY5.1 mAb, PE-conjugated anti-TCR Vα2 mAb, FITC-conjugated anti-TCR Vβ5.1/5.2 mAb, PE-Cy5-conjugated anti-CD4 mAb, and allophycocyanin-conjugated anti-CD8α mAb (all mAbs from BD Pharmingen). Endogenous NP_{366}-specific T cell responses were detected by staining with D^bNP_{366} tetramers, prepared as described previously (25). Propidium Iodide was added to select for live cells. Data acquisition and analysis was done on a FacsCalibur (Becton Dickinson) using CellQuest software. Blood glucose levels in RIP-OVA^{high} mice were monitored by Accu-Check Compact (Roche Diagnostics) measurement. Mice were considered diabetic when blood-glucose levels were 20 mM or above. For long-time follow-up, diabetic mice were treated with s.c. insulin implants according to the manufacturer's protocol (LinShin Canada).

In vivo cytotoxicity assay

Splenocytes from naive RIP-OVA^{high} mice were labeled with either 0.1 μM CFSE or 1 μM CFSE and the cells labeled with 1 μM CFSE were subsequently pulsed with 10 μM SIINFEKL peptide for 1 h at 37°C. After Ag loading, cells were washed three times and pulsed and unpulsed cells were subsequently injected into mice in a 1:1 ratio at a total of 1×10^7 cells per mouse. After 6 h, spleen cells were isolated and the ratio of CFSE^{high}/CFSE^{low} cells was determined by flow cytometry. The percentage Ag-specific cytotoxicity was determined as follows: $100 - ([\%$ peptide pulsed in infected / % unpulsed in infected) / (% peptide pulsed in naive / % unpulsed in naive)] $\times$ 100).

Barcode analysis of T cell responses

T cells transduced with the OT-I TCR were cotransduced with a retroviral "barcode" library (diversity of >3,000), in which each library member contains a unique stretch of semi-random DNA that can be used to perform clonal analyses of cell populations (K. Schepers, E. Swart, J. van Heijst, C. Gerlach, M. Castrucci, D. Sie, M. Heimerikx, A. Velds, R. Kerkhoven, and R. Arens, submitted for publication). Transductions were performed under conditions in which each TCR-transduced T cell contains approximately one barcode. In short, B6 splenocytes were retrovirally transduced with the OT-I TCR and a pMX barcode library that encodes a GFP marker gene. Transduced cells were sorted for expression of GFP, Vα2, and CD8. Individual mice were injected with a mixture of 2×10^3 barcode labeled OT-I transduced T cells and 1×10^5 nonlabeled OT-I transduced T cells. After induction of primary, or primary and secondary in vivo expansion, T cells were harvested from spleen suspensions. Subsequently, barcodes were recovered by PCR and labeled with Cy3 and Cy5 dyes, and the diversity of T cell populations was determined by hybridization on barcode microarrays (K. Schepers, E. Swart, J. van Heijst, C. Gerlach, M. Castrucci, D. Sie, M. Heimerikx, A. Velds, R. Kerkhoven, and R. Arens, submitted for publication). To quantify the number of participating T cell clones, the number of barcodes with a signal above background with a probability of <0.01 was determined (K. Schepers, E. Swart, J. van Heijst, C. Gerlach, M. Castrucci, D. Sie, M. Heimerikx, A. Velds, R. Kerkhoven, and R. Arens, submitted for publication). The cut-offs used for this quantification are indicated by the horizontal and vertical dividers in the dot plots.

Results

In vivo persistence and long-term reactivity of OT-I TCR transduced T cells

In prior studies, the in vivo behavior of TCR-modified T cells has been examined in settings where the TCR-modified T cells received antigenic stimulation directly after infusion. However, in settings of minimal residual disease the capacity of infused TCR modified T cells to persist in vivo without antigenic stimulation is likely to be important. To determine the capacity of TCR-transduced T cells for long-term in vivo persistence without Ag encounter, B6 splenocytes (LY5.2$^+$) were retrovirally transduced with the chicken OVA$_{257–264}$-specific OT-I TCR and were injected into naive B6LY5.1/5.2 F_1 mice. In all experiments, relatively low numbers of TCR-transduced cells were given (1×10^5), to allow extrapolation of the data toward clinically realistic protocols. Following adoptive transfer, mice were given a viral challenge with WSN-OVA, a recombinant influenza virus encoding the OVA$_{257–264}$ epitope, at 0, 3, 10, or 26 wk after adoptive transfer. Following

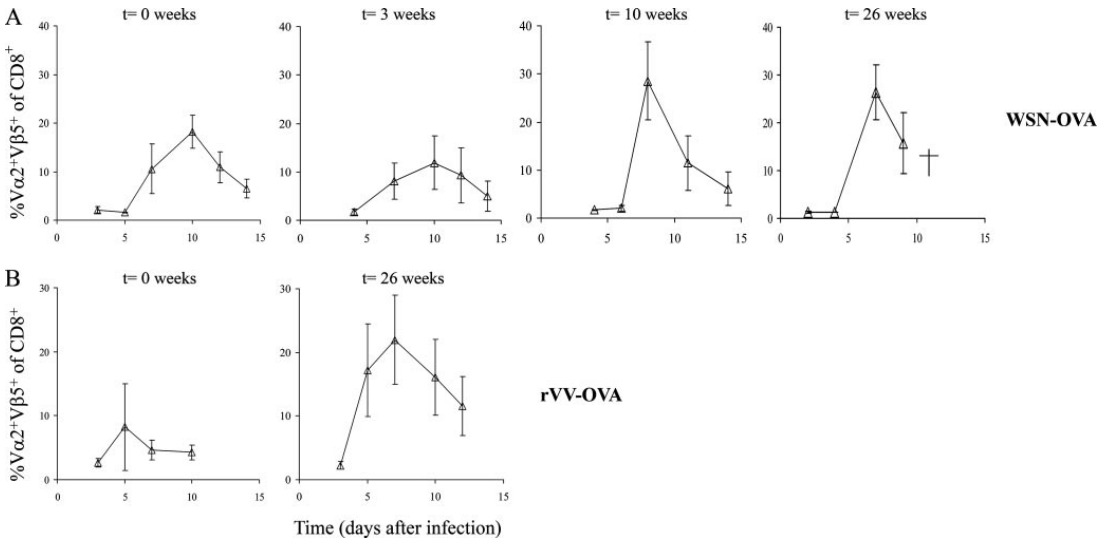

FIGURE 1. In vivo persistence of Ag responsive TCR transduced T cells. *A*, B6Ly5.1/5.2 mice ($n = 5$) received infusions of 1×10^5 OT-I TCR transduced B6 (Ly5.2) splenocytes. Mice were infected with the recombinant WSN-OVA influenza virus at the indicated time points post T cell infusion (t). T cell responses were measured in peripheral blood samples taken at the indicated time points post infection. *B,* As in *A,* but viral challenge was performed by infection with recombinant vaccinia virus encoding GFP-OVA (rVV-OVA) to avoid T cell-dependent virus induced pathology. Data represent group averages ± SD.

viral challenge, expansion of the gene-modified T cells in peripheral blood was measured using mAbs against LY5.1, CD8, and TCR Vα2 and Vβ5. Ag-driven expansion of TCR-modified T cells was observed both in mice challenged with Ag directly after adoptive transfer and in mice that had received T cell infusions between 3 wk and 6 mo before challenge (Fig. 1*A*). Remarkably, rather than the reduction in the magnitude of T cell responses that would be expected if T cell persistence was poor, there was a trend toward more rapid and pronounced T cell responses over time, in particular in the groups of mice that were challenged at 10 and 26 wk post transfer (average T cell responses at day 7 post infection: 0 wks: 18%, 26 wks: 26%, $p = 0.042$) Notably, three of five mice challenged at 26 wk post infusion had to be euthanized before completion of the experiment because of severe pulmonary distress, at which time point the experiment was terminated. As prior data have demonstrated that high-level cytotoxic T cell responses against influenza A epitopes can be fatal due to T cell mediated pulmonary damage (26, M. Coccoris, unpublished data), these data seemed consistent with an increasing rather than a lessening proliferative capacity of TCR-modified T cells upon prolonged in vivo presence. To test the possibility that T cell responses mounted by TCR-modified T cells become more pronounced after prolonged in vivo presence in an independent system, mice were infused with OT-I TCR-modified T cells and challenged at 0 or 6 mo with rVV-OVA. Even though OT-I-modified T cell responses in mice challenged directly after T cell infusion were mediocre in this experiment, marked T cell responses were seen in recipients that were challenged 6 mo after infusion of the cells (Fig. 1*B*). These findings show that without antigenic stimulation, TCR gene modified T cells can persist and remain Ag-responsive for months after cell transfer.

Secondary responses of TCR modified T cells

To subsequently address whether TCR gene-modified T cells can mount secondary responses in vivo, B6 LY5.1/5.2 mice were injected with 1×10^5 OT-I TCR transduced T cells and mice were challenged with WSN-OVA. Six to 8 wk after primary Ag encounter, mice were rechallenged with an increased dose (1000-fold) of the identical virus. Although a prominent T cell response of the OT-I TCR modified T cells was apparent during primary Ag encounter (peak average of 17% of total $CD8^+$ cells), no significant expansion of OT-I modified T cells could be detected upon re-challenge (Fig. 2, *left* and *middle panels*). This lack of expansion of TCR-modified T cells upon secondary Ag encounter was not due to rapid clearance of WSN-OVA by pre-existing Abs, as T cells specific for the influenza A $NP_{366-374}$ epitope present in the viral backbone of WSN-OVA were detected at a substantial frequency (Fig. 2, *right panel*). Thus, in a homologous prime-boost setting, the capacity of TCR-modified T cells to participate in a secondary T cell response was negligible. To determine whether this lack of an appreciable secondary response is an intrinsic limitation of TCR modified T cells or only observed in a homologous prime-boost setting, we evaluated secondary expansion of OT-I TCR transduced T cells upon primary challenge with rVV-OVA and rechallenge with WSN-OVA. In this heterologous prime-boost regimen, a clear expansion of OT-I TCR modified T cells is observed during secondary infection and the kinetics of expansion of the TCR transduced T cells upon secondary infection (rVV-OVA $\rightarrow$ WSN-OVA) are similar to those observed upon primary WSN-OVA infection (Figs. 1 and 3*A*). Notably, robust responses of TCR-modified T cells are also seen when mice receive a heterologous viral challenge 1 year or 18 mo after the primary infection (Fig. 3*B*).

The above data indicate that TCR gene-modified T cells that are specific for a foreign Ag can persist and participate in secondary T cell responses in vivo. However, TCR gene therapy is primarily developed as a strategy to generate a T cell repertoire that is reactive with defined self-Ags. To establish whether TCR-modified T cells that are specific for a self-Ag can also form a long-lived Ag-responsive T cell population, we examined secondary Ag responsiveness of OT-I TCR-transduced T cells in RIP-OVA^{high}

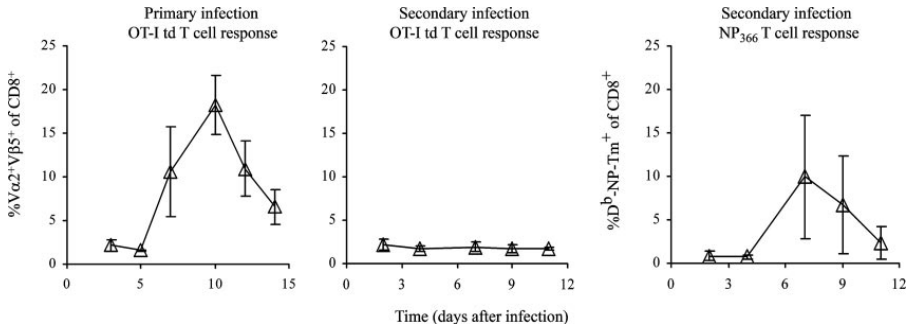

FIGURE 2. Absence of secondary OT-I-transduced T cell responses upon homologous viral challenge. B6Ly5.1/5.2 mice ($n = 5$) received infusions of 1×10^5 OT-I TCR transduced B6 (Ly5.2) splenocytes and were subsequently challenged by WSN-OVA infection. Six to 8 wk after primary infection, mice were rechallenged by infection with a 1000-fold increased dose of WSN-OVA. T cell responses were measured in peripheral blood samples taken at the indicated time points post viral challenge. Data represent group averages ± SD.

mice. In these mice, OVA is expressed in pancreatic β cells and, as a consequence, endogenous CD8 T cell responses against the $OVA_{257–264}$ epitope are below the level of detection (9). RIP-OVA^{high} mice were infused with OT-I TCR-transduced T cells and infected with rVV-OVA (Fig. 4*A*). Six to 8 wk after the primary response, mice were rechallenged with WSN-OVA and OT-I modified T cell responses in peripheral blood were monitored. Using this heterologous prime-boost strategy, a robust expansion of OT-I modified T cells was observed upon re-challenge and this expansion was comparable to that observed in WT mice (Fig. 3*A* and 4*A*). These data indicate that, at least in this mouse model, TCR gene modified T cells that are directed against an Ag for which an endogenous repertoire is lacking can persist in vivo and react to secondary Ag encounter.

Dissecting the effect of mitogenic stimulation and TCR gene transfer on primary and secondary Ag responsiveness in vivo

Memory T cell responses are generally described as being more rapid and of a higher magnitude. However, the kinetics and magnitude of the secondary T cell responses of TCR-modified T cells (Figs. 3*A* and 4*A*) are comparable to those observed upon primary Ag encounter of the same recombinant virus (Fig. 1*A*). The disparity between these data and the generally held view on primary

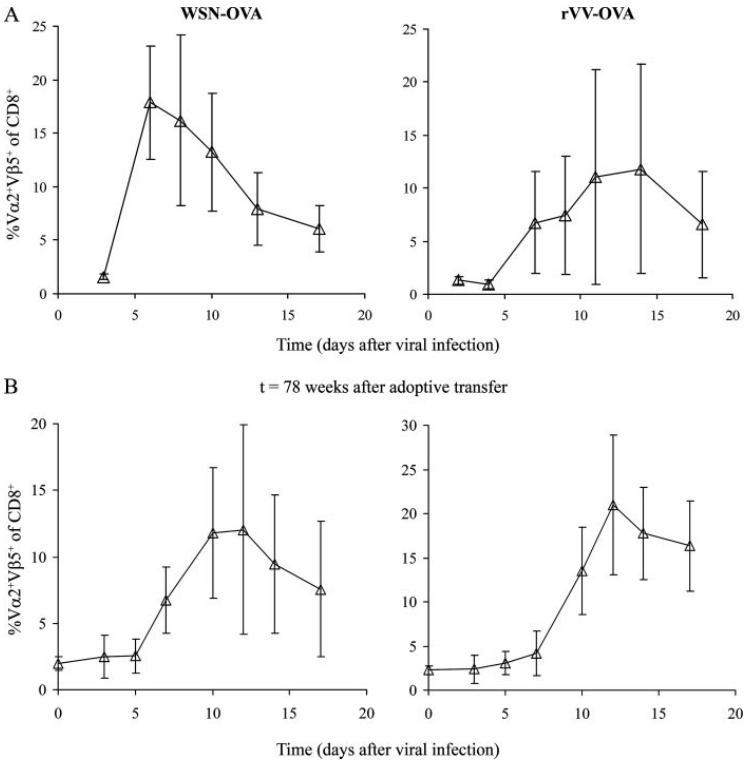

FIGURE 3. Secondary OT-I-transduced T cell responses upon heterologous viral challenge. *A,* B6Ly5.1/5.2 mice ($n = 5$) received infusions of 1×10^5 OT-I TCR-transduced B6 (Ly5.2) splenocytes and were subsequently challenged by rVV-OVA infection. Six to 8 wk after primary infection, mice were rechallenged by infection with WSN-OVA. T cell responses were measured in peripheral blood samples taken at the indicated time points post primary (*left*) or secondary (*right*) challenge. Data represent group averages ± SD. *B,* B6 Ly5.1/5.2 mice received infusions of 1×10^5 OT-I TCR-transduced B6 (Ly5.2) splenocytes. Mice were infected with rVV-OVA directly post T cell infusion ($n = 5$, *left panel*) or at wk 26 post cell infusion ($n = 7$, *right panel*). At 78 wk post T cell infusion, a rechallenge with WSN-OVA was given. Depicted are peripheral blood T cell responses upon secondary infection. Data represent group averages ± SD.

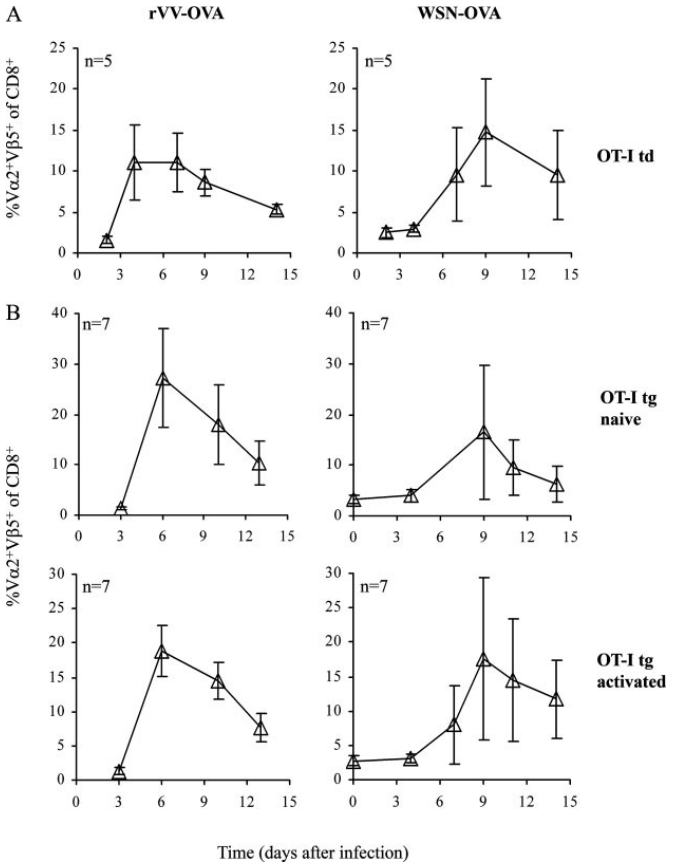

FIGURE 4. Primary and secondary TCR transduced and TCR transgenic T cell responses in mice lacking an endogenous Ova-specific T cell repertoire. *A,* RIP-OVA^{high} mice ($n = 5$) received infusions of 1×10^5 syngeneic OT-I TCR transduced splenocytes (OT-I td). *B,* RIP-OVA^{high} mice ($n = 7$) received infusions of either 1×10^5 naive OT-I transgenic cells (*top panel*, OT-I tg naive) or 1×10^5 pre-activated OT-I transgenic cells (*bottom panel*, OT-I tg activated). Mice were subsequently challenged by rVV-OVA infection. Six to 8 wk after primary infection, mice were rechallenged by WSN-OVA infection. T cell responses were measured in peripheral blood samples taken at the indicated time points post primary (*left*) or secondary (*right*) challenge. Data represent group averages ± SD.

and memory T cell responses could be due to several factors. First, the nonspecific T cell stimulation that is used for retroviral gene transfer may limit the capacity of these T cells to form T cell memory. Second, current TCR gene transfer procedures are based on the modification of pre-existing T cells that express endogenous TCR chains. The reduced expression of the introduced TCR or the formation of mixed dimers could possibly limit the competitive ability of TCR modified T cells to survive during the memory phase, or to mount a secondary response. Third, the observation that primary and secondary responses of TCR modified T cells are comparable in kinetics and magnitude may not so much reflect a reduced ability to mount secondary responses but rather an increased ability to mount rapid and strong primary T cell responses. To address these issues, we compared primary and secondary responses of TCR-modified T cells (Fig. 4*A*) to those of naive OT-I transgenic T cells and Con A stimulated OT-I transgenic T cells (Fig. 4*B*). Primary T cell responses were somewhat reduced in recipients of Con A activated OT-I transgenic T cells as compared with naive OT-I transgenic T cells (average peak primary T cell responses of 19 and 27% for recipients of activated and naive T cells respectively, $p = 0.014$). However, secondary T cell responses were comparable between the two groups (average peak secondary T cell responses of 17% for both recipients of naive and activated OT-I transgenic T cells). Importantly, the kinetics and magnitude of secondary responses in recipients of TCR transgenic T cells were comparable to those observed in recipients of TCR-transduced T cells (Figs. 1, 4*A*, and 4*B*). These data indicate that the capacity of TCR-modified T cells for long-term persistence after Ag challenge is similar to that of unmanipulated TCR transgenic T cells. Consequently, both the in vitro activation procedure and the fact that TCR-modified T cells harbor both endogenous and exogenous TCR chains appear to have a minimal effect on their capacity for long-term and repetitive in vivo function.

In vivo functionality of TCR-transduced T cells during secondary expansion

The functionality of TCR-modified T cells during primary T cell responses in RIP-OVA^{high} mice can be assessed by analyzing the development of type I diabetes as a consequence of destruction of pancreatic β cells (9). Primary T cell responses in recipients of both TCR transgenic and TCR modified T cells resulted in destruction of insulin producing cells in 100% of the mice (data not shown). As the development of type I diabetes is irreversible in this model, the use of this parameter as a read-out for T cell function during secondary responses is precluded. To assess the functionality of TCR-transduced T cells during secondary Ag-specific expansion, we therefore analyzed their capacity to destruct Ag-loaded target cells in vivo. To this purpose, a 1:1 mixture of unpulsed CFSE^{low} RIP-OVA^{high} splenocytes and Ova peptide pulsed CFSE^{high} RIP-OVA^{high} splenocytes was injected at the peak of the secondary T cell response. Six hours after injection, spleen cells were isolated and the ratio of CFSE^{low} and CFSE^{high} cells was

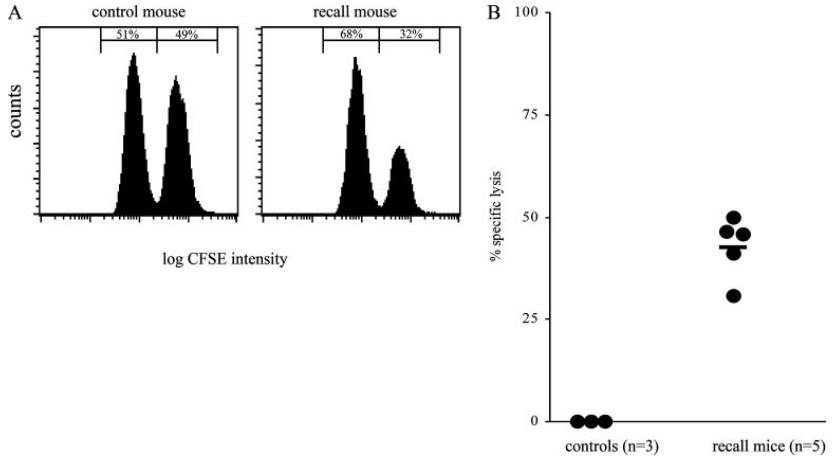

FIGURE 5. Long-term in vivo function of TCR modified T cells. RIP-OVA^{high} mice ($n = 5$) received infusions of 1×10^5 syngeneic OT-I TCR transduced splenocytes and were subsequently challenged by rVV-OVA infection. Six to 8 wk after primary infection, the mice were rechallenged by WSN-OVA infection. CFSE^{low} labeled unpulsed splenocytes and CFSE^{high} labeled SIINFEKL-pulsed splenocytes were injected at a 1:1 ratio around the peak (day 9) of the secondary response. As a control, RIP-OVA^{high} mice that did not receive an adoptive transfer of transduced cells were used. *A,* Flow cytometric analysis of spleen cells of a control mouse (*upper left panel*) and a recipient of OT-I TCR transduced T cells (*upper right panel*) 6 h after transfer of CFSE-labeled cells. *B,* Percentage specific lysis of Ova$^+$CFSE^{high} cells in a 6-h in vivo cytotoxicity assay. Closed dots indicate values in individual mice, bars represent group averages.

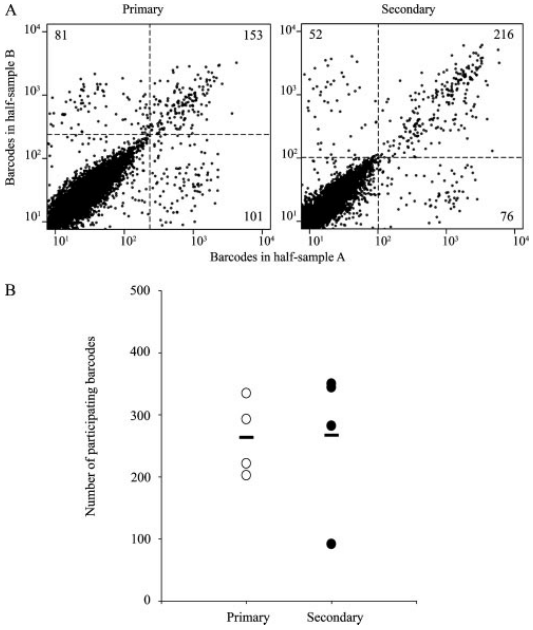

FIGURE 6. Polyclonal responses of TCR transduced T cells. *A,* B6Ly5.1/5.2 mice ($n = 4$ per group) received infusions of 1×10^5 OT-I TCR transduced, plus 2×10^3 barcode-labeled OT-I TCR-transduced B6 (Ly5.2) T cells (*left panel*) and were either challenged by rVV-OVA infection, or received prime-boost vaccinations (rVV-OVA–WSN-OVA) with a 6–8 wk interval. At the peak of both the primary and secondary response, spleens were collected and isolated barcode-labeled cells were split into two identical half-samples. To determine sampling efficiency, barcodes from these half-samples were independently amplified by PCR, labeled with Cy-3 and Cy-5 dyes, and cohybridized to the barcode-microarray. Dot plots depict fluorescence intensities of both half-samples. Values indicate the number of barcodes detected above background in each quadrant. *B,* Total number of participating barcodes for both primary and secondary response groups. Circles depict individual mice, bars depict group averages.

determined (Fig. 5*A*). As expected, no specific killing of Ova-pulsed cells was observed in naive RIP-OVA^{high} mice (average specific lysis 0%, $n = 3$). In contrast, in recipients of TCR-modified T cells that were rechallenged with Ag 6–8 wk after the primary response, efficient lysis of Ova-loaded target cells was observed (average specific lysis 43%, $n = 5$) (Fig. 5*B*). These data indicate that TCR-modified T cells that respond to secondary Ag challenge in a self-tolerant setting are functional, as revealed by in vivo kill of Ag-positive target cells.

Secondary responses of TCR-modified T cells are polyclonal

To determine whether the adoptively transferred T cells participating in a secondary response are polyclonal or derived from a limited set of T cells, we labeled OT-I TCR-transduced T cells with unique identifiers ("barcodes") by retroviral infection. To this purpose, we made use of a retroviral plasmid library, containing >3,000 unique sequences, all coupled to a GFP marker gene (K. Schepers, E. Swart, J. van Heijst, C. Gerlach, M. Castrucci, D. Sie, M. Heimerikx, A. Velds, R. Kerkhoven, and R. Arens, submitted for publication). After adoptive transfer of 2×10^3 barcode-labeled TCR transduced T cells plus 1×10^5 non-barcode-labeled TCR-transduced T cells, recipient mice were challenged with rVV-OVA. Analysis of barcode diversity was subsequently performed at the peak of the primary response for one group of mice (Fig. 6*A*, *left panel*). A second group of mice was rechallenged with WSN-OVA 6 wk later and barcode diversity was determined at the peak of the secondary T cell response (Fig. 6*A*, *right panel*). When barcode diversity is compared between primary and secondary T cell responses, the number of clones with detectable participation is essentially identical. Thus, secondary T cell responses of TCR-modified T cells remain highly polyclonal and are not driven by expansion of one or a few T cell clones (Fig. 6*B*).

Discussion

TCR gene transfer allows the generation of T cells with a defined Ag specificity, even when this specificity is lacking in the endogenous T cell repertoire. This strategy can be considered analogous

to the clinical use of Abs such as Herceptin and Rituxan that recognize tumor-associated self-Ags for which the human immune system is tolerant. However, while a substantial amount of data has been generated on the pharmacokinetics of therapeutic Abs, no data is available on the in vivo persistence and maintenance of function of TCR-modified T cells.

A number of issues may conceivably limit the in vivo persistence of TCR-modified T cells. After adoptive transfer, the infused gene-modified T cells may be recognized and targeted by the immune system of the recipient. Alternatively, the long-term function of TCR-modified T cells may be reduced as a consequence of the retroviral modification procedure. Clinical studies using TIL infusion have demonstrated that the long-term persistence of adoptively transferred cells correlates with clinical responses (3). As a durable engraftment of TCR-modified T cells is likewise expected to be required, in this study, we examined the function of TCR-modified cells for up to 18 mo after adoptive transfer. In these experiments, mice were infused with 1×10^5 TCR-modified T cells, the equivalent of 1×10^9 TCR-modified T cells in humans (assuming a $CD8^+$ T cell pool of 2×10^7 and 2×10^{11} in mice and humans, respectively), slightly below the average dose used by Morgan et al. (10). The two main conclusions from these experiments are that retrovirally TCR-transduced T cells can persist for prolonged periods in vivo without a requirement for Ag-specific stimulation and that TCR-modified T cells provide a capacity for secondary T cell responses that is comparable to that of nonmanipulated TCR transgenic cells.

Ag-reactive TCR-modified T cells persisted for periods of at least 18 mo after adoptive transfer in the current experiments. This indicates that, at least in this system, the infused cells are not rendered immunogenic by TCR gene modification and that the retroviral activation procedure does not impair the capacity of TCR-modified T cells for long-term engraftment. A further concern in TCR gene transfer has focused on the possibly of limited stability of TCR transgenes in gene modified T cells. Specifically, mouse studies have shown that silencing of retroviral transgenes in hematopoietic cell types may occur (27). Furthermore, transcription from the retroviral LTR was shown to be reduced in quiescent T cells (28–29). The current data demonstrate that, at least for murine T cells, such inactivation of transgene expression is insufficiently strong or frequent to prevent robust T cell responses of TCR-modified T cells upon introduction of Ag at later time points. By examining clonal diversity in TCR-modified T cell responses, it was also shown that secondary responses of TCR-modified T cells are not driven by a limited set of clones. These data provide some evidence that retroviral modification of T cells may not lead to the preferential outgrowth of cells that carry integrations near genes controlling proliferative capacity that has been observed for gene-modified hematopoietic stem cells (30). In this regard, it is also interesting to note that in mice that received OT-I modified T cells, no rise in the frequency of $V\alpha2^+V\beta5^+$ T cells was seen during a follow-up of 18 mo, unless Ag was provided (Fig. 3*B*, data not shown). These data suggest that in this murine model, the risk for cellular transformation due to retroviral modification with TCR-encoding vectors may be low. However, studies with a longer follow-up will clearly be required.

Interestingly, the magnitude of TCR-modified T cell responses appears to increase upon prolonged periods of in vivo persistence. The in vitro activation of T cells that is required for retroviral gene transfer leads to phenotypic maturation, including down-regulation of CD62L expression. As a consequence, TCR-modified T cells may have a reduced capacity for lymph node entry, thereby reducing Ag responsiveness shortly after infusion (31). Conceivably, acquisition of a central memory T cell phenotype, including re-expression of CD62L, could then explain the enhanced responsiveness of the infused cells over time (32).

In a second set of experiments, it was demonstrated that TCR-modified T cells have the capacity to react to secondary Ag encounter in vivo. To achieve a productive secondary response of TCR-modified T cells, it was essential to use a heterologous prime-boost regime. This indicates that, even in a setting where the T cell repertoire is highly biased at the start of the primary response, immunodominance of other T cell specificities (33) can interfere with activation of the infused cell population during secondary Ag encounter. Notably, the kinetics and magnitude of secondary T cell responses obtained in these prime-boost experiments were not increased as compared with primary T cell responses of the same TCR-modified T cells. This contrasts with the classical description of memory T cell responses, typically characterized by an accelerated expansion and increased magnitude (34, 35). Importantly, this difference with classical memory T cell responses is not due to the process of TCR introduction, as the same phenomenon is observed after infusion of nonmodified TCR transgenic T cells (Fig. 4). Thus, the observed lack of increased T cell responsiveness in recall responses is likely due to the fact that a large T cell pool is already available for Ag recognition at the start of the primary T cell response, as a consequence of the adoptive transfer.

Finally, to analyze a possible effect of the TCR gene transfer procedure on the capacity for Ag-specific proliferation of the adoptively transferred cells, we compared responsiveness of TCR transduced cells with that of quiescent or activated TCR transgenic T cells with the identical specificity. Results of these experiments show that primary T cell responses of TCR modified T cells are some 2–3-fold reduced as compared with those of naive TCR transgenic cells. As discussed above, this may at least in part be due to a detrimental effect of the in vitro T cell activation procedure on the ability of cells to participate in the Ag-specific T cell response. In line with this, primary (but not secondary) T cell responses of TCR transgenic cells are also reduced to some extent by in vitro T cell activation. These data provide indirect support for the preclinical and clinical testing of lentiviral vector systems that do not require mitogenic T cell stimulation.

In conclusion, the current data demonstrate that TCR transduced T cells remain present in the host after adoptive transfer and can be activated in vivo up to 18 mo after infusion. Furthermore, the capacity of TCR gene-modified T cells to react upon secondary Ag encounter in vivo, is comparable to that of T cells that have not undergone in vitro modification. These features, together with the previously described capacity for recognition of defined tumor-associated self-Ags, provide a clear incentive for the further clinical testing of TCR modified T cells in oncology.

Disclosures

The authors have no financial conflict of interest.

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Chapter 5

αβ T cell receptor transfer to γδ T cells generates functional effector cells without mixed TCR dimers

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Accepted for publication in the Journal of Immunology

αβ T cell receptor transfer to γδ T cells generates functional effector cells without mixed TCR dimers *in vivo*

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Keywords: Rodent, T Cells, T Cell Receptors, Gene Therapy

The successful application of T cell based immunotherapeutic applications depends on the availability of large numbers of T cells with the desired antigen specificity and phenotypic characteristics. Engineering of TCR transferred T lymphocytes is an attractive strategy to obtain sufficient T cells with an antigen specificity of choice. However, the introduction of additional TCR chains into T cells leads to the generation of T cells with unknown specificity, due to the formation of mixed dimers between the endogenous and introduced TCR chains. The formation of such potentially auto-aggressive T cells may be prevented by using γδ T cells as recipient cells, but the *in vivo* activity of such TCR-engineered γδ T cells has not been established. In the present study we have investigated the *in vivo* functionality of TCR transduced γδ T cells, in particular their antigen specific proliferative capacity, antigen specific reactivity, *in vivo* persistence and their capacity to mount recall responses. The results demonstrate that αβTCR engineering of γδ T cells forms a feasible strategy to generate antigen specific effector T cells that do not express mixed TCR dimers. In view of increasing concerns on the potential autoimmune consequences of mixed TCR dimer formation, the testing of αβTCR engineered γδ T cells in clinical trials seems warranted.

Introduction

Several lines of evidence suggest that T cell based adoptive immunotherapy forms an attractive approach for the treatment of various malignancies. Infusion of donor

lymphocytes for the treatment of hematological malignancies has been shown to result in 20-80% complete remissions depending on the nature of the leukemia(1-5). Successful adoptive immunotherapy based on adoptive transfer of *ex vivo* expanded tumor infiltrating lymphocytes in conditioned recipients has been demonstrated for metastatic melanoma(6,7).

Although promising, the complex isolation methods of T cells with the desired antigen specificity and laborious and time consuming tissue culture procedures are likely to prevent the widespread application of these approaches. We and others have demonstrated that these difficulties can be overcome by an alternative strategy, namely the transfer of T cell specificity by genetic introduction of T cell receptor (TCR) alpha and beta chains into T lymphocytes(8-15). Since T cell specificity is exclusively determined by the TCR, genetic transfer of TCR chains to T cells generates T cells with the desired antigen specificity. The potential *in vivo* efficacy of TCR-transferred T cells has been demonstrated in mouse models(14-17). As shown in these various murine models, TCR-transferred T cells can be activated by antigen *in vivo*, home to effector sites, and can break tolerance against defined self antigens. Importantly, TCR gene transfer can largely halt tumor progression in a spontaneous prostate carcinoma model, under conditions in which vaccination is without effect(18). Recently, in a clinical phase 1 study autologous peripheral blood derived lymphocytes transduced with a Mart-1 specific TCR were reinfused in patients with metastatic melanoma after lymphodepleting chemotherapy(19). Persistence of the TCR modified T cells was observed for more than 2 months and 2 of 15 patients showed objective tumor regression illustrating that, although optimization is required, TCR gene transfer is feasible in a clinical setting.

A potential complication in TCR gene transfer is the formation of mixed TCR dimers, that occurs when introduced TCR alpha and beta chains pair with the endogenous TCR alpha and beta chains(20). As the specificity of these mixed TCR dimers is unknown, the formation of unwanted autoreactivities cannot be excluded(12,21). Importantly, while no evidence for mixed dimer induced autoimmunity was observed in earlier murine experiments and a first phase I trial(14-17,19), more recent data in mouse model systems indicate that under conditions of aggressive conditioning, severe mixed dimer-dependent autoimmune pathology can occur (G. Bendle, pers. comm).

Prior work has illustrated that the γ and δ TCR chains that are expressed by γδ T cells cannot form heterodimers with α and β TCR chains(22,23). We previously demonstrated that the redirection of γδ T cells instead of αβ T cells allows TCR gene transfer without the formation of mixed TCR dimers(24). Specifically, *in vitro* experiments showed that after retroviral transfer of MHC class I or MHC class II restricted TCRs fully functional, redirected human γδ effector cells were obtained capable of lysing leukemic cells in an antigen specific manner. TCR gene transfer into γδ T cells requires either the co-transfer of the CD4 or CD8 co-receptor, or the use of co-receptor independent TCRs, as the majority of γδ T cells lack the expression of these co-receptors(25,26). However, as demonstrated previously, the joint introduction of TCR and co-receptor genes can be achieved readily(16,24).

A substantially greater concern is formed by the lack of information on the *in vivo* capacities of TCR modified γδ T cells. As compared to αβ T cells, relatively little is known on the *in vivo* survival and effector functions of γδ T cells. Furthermore, while γδ T cells can be detected in peripheral blood and spleen, this T cell subset mainly

resides in epithelial sites and this could affect the capacity of αβTCR-modified γδ T cells to react to antigen(27-31).

To address these issues, we investigated the *in vivo* function of αβTCR-modified γδ T cells in a mouse model. Our data demonstrate that αβTCR gene transfer into γδ T cells can be used as a safe method to generate functional antigen specific effector cells for adoptive immunotherapy.

Materials and Methods

Mice and γδ T cells

C57BL/6 (B6), B6 Ly5.1^{+}, B6 Ly5.1/2^{+}, B6 Ly5.2^{+}, and RIP-OVA^{hi} mice(32) were obtained from the animal department of the Netherlands Cancer Institute. All animal experiments were carried out in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of the Netherlands Cancer Institute (DEC).

To isolate spleen derived γδ T cells from B6 Ly5.1^{+}, B6 Ly5.1/2^{+}, or B6 Ly5.2^{+} mice, splenocytes were stained with PE-conjugated anti-TCRγδ specific mAbs (BD Biosciences, MountainView, CA (BD)), anti-PE Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and γδ T cells were enriched by AutoMACS (Miltenyi Biotec) according to the manufacturers protocol (>50% purity). Subsequently, the recovered cells were further purified using a Vantage (Becton-Dickinson[BD], San Jose, CA) fluorescence activated cell sorter. Purity of the thus obtained γδ T cell population was >99%.

Construction of the retroviral vectors and production of the retroviral supernatant

The pMX vector encoding the TCRα and TCRβ chains of the OT-I TCR separated by an internal ribosome entry site (IRES) and the pMX vector encoding the murine CD8α and CD8β chains separated by an IRES have been described previously(16,17). Retroviral supernatants were obtained by transfection of Phoenix-E packaging cells with the indicated retroviral vectors in combination with pCLEco(33), using the FuGENE^{TM} 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany). Retroviral supernatants were obtained 48 hours after transfection and used for transduction of purified γδ T cells.

Retroviral transfer of the OT-I αβTCR and coreceptors to γδ T cells

Purified γδ T cells were stimulated in 24 well plates for 24 hours in RPMI 1640 medium (Life Technologies BV, Scotland) supplemented with 8% fetal calf serum (BioWhittaker, Belgium), penicillin (100 U/ml) and streptomycin (100 μg/ml, Boehringer Mannheim, Germany) in the presence of irradiated autologous feeder cells, Concanavalin A (2 μg/ml, Calbiochem, Darmstadt, Germany) and IL-7 (5 ng/ml, Santa Cruz Biotechnology, Santa Cruz, CA).

Non-tissue culture-treated 24 well plates (Becton Dickinson) were coated with 0.5 ml of 30 μg/ml recombinant human fibronectin fragment CH-296 (RetroNectin^{TM}; Takara, Otsu, Japan) at room temperature for 2 hours. The CH-296 solution was removed and replaced with 0.5 ml 2% bovine serum albumin (Sigma, St. Louis, MO) in PBS for 30 min at room temperature. The γδ T cells were plated on RetroNectin^{TM} coated plates ($0.3x10^6$ cells/well) in 0.5 ml of retroviral supernatant and cultured at 37ºC for 24 hours. For *in vivo* assays, the cells were washed once in Hank's balanced

salt solution (HBSS, Gibco), resuspended in HBSS and injected in mice intravenously.

Influenza A and recombinant vaccinia virus

For live influenza infections, anesthetized mice were infected by intranasal administration of 50 µl of HBSS (Life Technologies, Grand Island, NY) containing 200 plaque forming units (PFU) of influenza A/WSN/33 (WSN)-OVA(I)(34) virus that expresses the $OVA_{257-264}$ epitope (WSN-OVA), or 0.5 hemagglutinating units (HAU) of influenza A/HK/2/68 virus. For recombinant vaccinia infections, $2x10^7$ PFU of vaccinia recombinant for GFP-$OVA_{257-264}$ (rVV-OVA) were injected intraperitoneally.

Peptides

The H-$2K^b$ binding peptides $OVA_{257-264}$ (SIINFEKL) and SV40 large $T_{404-411}$ (VVYDFLKC), the H-$2D^b$ binding peptides $NT_{366-374}$ (influenza A/HK/2/68: ASNENMDAM) and $PR_{366-374}$ (influenza A/PR/8/34: ASNENMETM) were synthesized by standard FMOC chemistry.

Flow cytometric analysis

For analysis of T cell responses, peripheral blood was drawn at the indicated time points. Erythrocytes were removed by incubation in erylysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) on ice for 15 min. Cells were stained with the indicated antibodies for 10-15 minutes at room temperature. The following mAbs: anti-TCRγδ FITC, anti-TCRγδ PE, anti-TCRVβ5.1 FITC, anti-TCRVα2 PE, anti-Ly5.1 PECy5, anti-Ly5.2 PECy5, and anti-CD8α APC were obtained from

Pharmingen (BD), anti-CD8β PE was obtained from Caltag Laboratories (Burlingame, CA). Before analysis, propidium iodide (1 μg/ml, Sigma) was added to select for propidium iodide-negative (living) cells.

Intracellular IFNγ staining

For intracellular IFNγ staining, cells were incubated in the presence of either 0.1 μg/ml OVA or 1 μg/ml SV40 peptide for 4 hours at 37°C, in the presence of Golgiplug (1 μl/ml, BD). Subsequently, cells were surface stained with FITC-conjugated anti-Ly5.2 (BD) and PE-conjugated anti-CD8α (Caltag) mAbs for 15 minutes on ice. After washing, cells were incubated in Cytofix/Cytoperm solution (BD) for 20 minutes on ice, washed and stained for intracellular IFNγ with APC-conjugated anti-IFNγ mAb (BD) on ice for 20 minutes.

***In vivo* cytotoxicity assay**

Splenocytes were prepared from RIP-OVA^{hi} mice and divided into two groups. Cells were labeled with 0.5 or 5 μmol/l carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA) for 20 min at 37°C. Cells were washed, kept on ice and subsequently the CFSE^{high} cells were pulsed with 0.1 μmol/l $OVA_{257-264}$ peptide for 1 h at 37°C. Five million cells from the CFSE^{low} and the CFSE ^{high} peptide pulsed cells were mixed together in equal proportions and injected intravenously into mice that had received γδ T cells transduced with the OT-I TCR and CD8 coreceptor, and had been infected with either WSN-OVA or influenza A/HK/2/68. Spleens were removed 5 h later and single-cell suspensions were generated for FACS analysis. The percentage of target cell killing was determined as: 100 – ((percentage of peptide-pulsed targets in WSN-OVA infected

recipients/percentage of unpulsed targets in WSN-OVA infected recipients)/(percentage of peptide-pulsed targets in influenza A/HK/2/68 infected recipients /unpulsed targets in influenza A/HK/2/68 infected recipients) x 100).

Results

Functional analysis of αβTCR transduced γδ T cells

To investigate the *in vivo* functionality of γδ T cells that have been redirected by the introduction of an αβ TCR, spleen derived γδ T cells were isolated by immunomagnetic bead isolation and subsequent FACS sorting (>99% purity). Purified γδ T cells were then stimulated with ConA and autologous irradiated feeder cells in the presence of IL-7, and after 24 h of stimulation retrovirally transduced with the OVA specific OT-I TCR. All TCR transductions were performed in combination with a retroviral vector encoding the CD8αβ coreceptor, as most γδ T cells lack expression of this co-receptor. The OT-I CD8 transduced γδ T cell population was infused intravenously ($1x10^5$ cells per mouse), and the mice then received an intranasal infection with either a recombinant influenza A strain that expresses the $OVA_{257-264}$ epitope (WSN-OVA) or a control influenza A strain (A/HK/2/68). Subsequently, the frequency of OT-I^+ $CD8^+$ γδ T cells in peripheral blood was determined by combined TCRγδ, TCR Vα2, Ly5.2 and CD8 staining at various time points post infection. In mice that were infected with WSN-OVA a significant accumulation of the OT-I modified γδ T cells was observed, with peak immune responses at day 9 post adoptive transfer (Fig. 1A middle panels). In contrast, in control mice that were infected with influenza A/HK/2/68 no significant numbers of OT-I modified γδ T cells could be detected (Fig. 1A top panel). The TCR$γδ^+$ TCR

Vα2$^+$ CD8$^+$ cell population that was detected in mice infected with WSN-OVA expressed the Ly5.2 marker present on the donor cells, and was hence fully donor-derived. Furthermore, the vast majority of Ly5.2$^+$ cells present in these mice expressed the γδ TCR, indicating that there was little if any contribution of the small amount (<1%) of co-transferred αβ T cells (Fig. 1A lower panel). These data demonstrate that γδ T cells that are engineered to express an MHC class I-restricted TCR are capable of antigen specific survival or proliferation upon interaction with antigen-expressing APC *in vivo*.

To assess whether this antigen-driven activation of OT-I CD8 transduced γδ T cells led to the acquisition of effector function, γδ T cells from WSN-OVA and control influenza-infected mice were compared for their ability to produce IFNγ *ex vivo*. At the peak of the response (day 9), peripheral blood derived lymphocytes were stimulated either with $OVA_{257-264}$ peptide or SV40 large $T_{404-411}$ control peptide. Subsequently, antigen specific intracellular IFNγ production was determined by flow cytometry. A substantial fraction of Ly5.2$^+$ OT-I TCR transduced γδ T cells that produced IFNγ was detected when lymphocytes derived from WSN-OVA infected mice were stimulated with OVA but not when stimulated with the control antigen SV40 (Fig. 1B). For comparison the percentage of endogenous IFNγ$^+$ Ly5.2$^-$ cells of the CD8$^+$ cells (reflecting OVA-specific αβ T cells) is shown. In control influenza-infected mice no IFNγ producing OT-I CD8 transduced γδ T cells were detected (data not shown).

In addition, total splenocytes of A/HK/2/68 infected and WSN-OVA infected mice were stimulated with OVA peptide and cultured for 14 days. Subsequently, the cultured splenocytes were restimulated with either SV40 or OVA peptide and

intracellular IFNγ production was determined (Fig. 1C). No Ly5.2^{+} cells were detected in influenza A/HK/2/68 infected mice, indicating that efficient recovery of αβ TCR transduced γδ T cells requires *in vivo* antigen encounter. In contrast, substantial numbers of the adoptively transferred Ly5.2^{+} OT-I CD8 transduced γδ T cells were found in cultures from WSN-OVA infected mice and a large fraction of these cells produced IFNγ after stimulation with OVA peptide. Collectively, these data indicate that γδ T cells modified with an αβ TCR exhibit the potential to undergo antigen-driven expansion and display effector functions following *in vivo* activation.

Self-antigen specific reactivity of αβTCR transduced γδ T cells

To investigate whether OT-I CD8 transduced γδ T cells can expand upon antigen specific stimulation in a situation where the endogenous T cell repertoire is tolerant towards the OVA antigen, OT-I CD8 transduced γδ T cells were infused in RIP-OVA^{HI} mice. In these mice ovalbumin is expressed in pancreatic β cells and no endogenous OVA specific cytotoxic and helper T cell responses are observed(35,36). Following infusion of OT-I CD8 transduced γδ T cells (5x10^{4} per mouse), the mice were infected either by intranasal application of influenza A/HK/2/68 or WSN-OVA, or by intraperitoneal injection of a recombinant vaccinia virus expressing GFP-OVA$_{257-264}$ (rVV-OVA). At various time points, the percentage of OT-I CD8 transduced γδ T cells in peripheral blood was monitored (Fig. 2). In mice that received OT-I CD8 transduced γδ T cells and were subsequently infected with either WSN-OVA or rVV-OVA, a substantial increase in αβTCR transduced γδ T cell number was observed, with a peak response of 8% of total CD8^{+} T cells in the latter group. Similar to the kinetics observed upon antigen-induced proliferation of TCR-

engineered αβ T cells(14,17), expansion was followed by a rapid contraction at the time of antigen clearance. As expected, no expansion of the Vα2$^+$ TCRγδ$^+$ CD8$^+$ T cell population was observed in mice that were infected with influenza A/HK/2/68. Thus, αβTCR-engineered γδ T cells can respond to *in vivo* antigen encounter in a situation where the endogenous T cell repertoire is tolerant towards the antigen of interest.

Analysis of intestinal sites for the presence of αβTCR engineered γδ T cells

A significant percentage of the CD8$^+$ lymphocytes present within the intestinal epithelium consists of γδ T cells(27-31). To investigate whether αβTCR transduced spleen derived γδT cells would preferentially home to intestinal epithelial sites upon intravenous infusion, the number of αβTCR transduced γδT cells in peripheral blood was compared with the number of αβTCR transduced γδT cells in the intra epithelial lymphocyte (IEL) and lamina propria-resident (LP) lymphocyte fractions. RIP-OVA^{HI} mice received either OT-I CD8 transduced γδ T cells, CD8 transduced γδ T cells ($2x10^5$ per mouse) or no T cells and were subsequently infected with WSN-OVA. As unmodified γδ T cells lack expression of CD8β(26), expression of CD8β was used to distinguish endogenous and adoptively transferred γδ T cells. The percentage of CD8β$^+$ γδ T cells in blood was monitored at various time points after the infection (Fig. 3A). Only in peripheral blood of mice that received γδ T cells transduced with the OT-I TCR in combination with the CD8 coreceptor CD8β$^+$ γδ T cells were detected. At the peak of the response (day 12) blood, IEL and LP lymphocyte fractions were analyzed by flow cytometric analysis. While in peripheral blood percentages of total γδ T cells up to 5% were observed, the percentage of total γδ T

cells in the IEL and LP reached levels up to 36% and 14%, respectively. In peripheral blood approximately 7.5% of the γδ T cells consisted of OT-I CD8 transduced γδ T cells (Fig. 3B). Analysis of IEL and LP derived T cells of the mice that received either OT-I CD8 transduced γδ T cells, or CD8 transduced γδ T cells or no T cells showed that no significant numbers of OT-I CD8 transduced γδ T cells could be detected in the intestine of the mice (Fig. 3C). This indicates that the OT-I CD8 transduced γδ T cells do not preferentially home to intestinal epithelial sites upon intravenous infusion.

***In vivo* persistence of αβTCR transduced γδ T cells**

To investigate whether infused OT-I CD8 transduced γδ T cells are able to persist *in vivo* and able to generate T cell memory, RIP-OVA^{HI} mice received an intravenous infusion of OT-I CD8 transduced γδ T cells ($4x10^5$) and subsequently an intranasal infection with WSN-OVA or influenza A/HK/2/68. At various time points the percentage of OT-I CD8 transduced γδ T cells in blood was monitored. Mice that received OT-I CD8 transduced γδ T cells and subsequently a WSN-OVA infection showed an increase in αβTCR transduced γδ T cell number from day 7 with a peak of the response at day 10 and subsequent contraction (Fig. 4). To test whether the antigen clearance preceding the observed contraction of the immune response was accompanied by cytolytic activity of the OT-I CD8 transduced γδ T cells, an *in vivo* cytotoxicity experiment was performed. Influenza A/HK/2/68 or WSN-OVA infected RIP-OVA^{HI} mice infused with OT-I CD8 transduced γδ T cells ($4x10^5$ per mouse) received CFSE^{low} labeled cells and OVA peptide pulsed CFSE ^{high} labeled cells intravenously. The percentages of direct antigen specific target cell lysis of OVA peptide pulsed cells after 5 hours varied from 18.5 to 24% (fig 5). As expected, no specific killing of OVA peptide pulsed cells was observed in influenza A/HK/2/68

infected mice. Thus, OT-I CD8 transduced γδ T cells are capable of antigen specific cytolytic activity *in vivo*. To test *in vivo* persistence, the generation of memory and the capacity of the OT-I CD8 transduced γδ T cell to mount a recall response, the mice that initially received an WSN-OVA infection were challenged by intraperitoneal injection of rVV-OVA after 12 weeks. Upon rVV-OVA challenge a strong increase in the number of OT-I CD8 transduced γδ T cells was observed in the peripheral blood of these mice leading to a recall response both in magnitude and kinetics comparable to the observed primary response (Fig. 4). Thus αβTCR modified γδ T cells are capable of prolonged *in vivo* persistence and αβTCR modified γδ T cells can mount efficient recall responses.

Discussion

In the current study we showed that γδ T cells modified with an αβTCR and the relevant coreceptor proliferated *in vivo* in an antigen specific manner and survived to mount efficient recall responses. The modified γδ T cells were found to reside in peripheral blood, and only low numbers were detected in the intestinal epithelial sites. Functional analysis indicated that αβTCR and CD8 transduced γδ T cells produced IFNγ upon antigen specific stimulation directly *ex vivo* and after *in vitro* stimulation. The currently most established strategy for TCR gene transfer, in which TCR genes are introduced into αβ T cells results in the formation of mixed TCR dimers due to pairing of endogenous and introduced TCRα and TCRβ chains (20). The specificity of the mixed TCR dimers is unknown and may be harmful, and therefore avoidance of the formation of mixed TCR dimers is desired. We propose TCR gene transfer into γδ

T cells instead of αβ T cells, which prevents the formation of mixed TCR dimers since γδTCR chains cannot pair with αβTCR chains(22,23).

As compared to earlier experiments in which the *in vivo* function of TCR modified αβT cells was investigated in murine models, TCR modified γδ T cells appear to have a somewhat lower proliferative capacity and *in vivo* effector function (17,37). This may in part be explained by the relatively low cell numbers used in this study. The γδ T cells isolated from spleen tissue underwent a stringent sorting procedure to render a >99% pure population, which unfortunately decreased the number of cells available for infusion. Furthermore, this selection process may also have affected the viability of the γδ T cells, reducing the proliferative capacity and therefore the effectiveness of the cells. This is in contrast to previous studies with TCR transduced αβ T cells, in which no sorting procedures were required(14,17). Importantly, it seems plausible that high numbers of TCR-modified γδ T cells can be generated with substantially greater ease in the human setting (see below).

Conflicting data exist on the capacity of γδ T cells to persist *in vivo*, to form long term immunological memory after an initial infection, and to subsequently mount a rapid recall response upon reinfection. While some murine studies showed a lack of effective immunity of γδ T cells to rechallenge(38-41), adoptive transfer of mouse γδ T cell lines to naïve recipients has been demonstrated to successfully restrict a malaria infection(42,43). Furthermore, in a nonhuman primate model γδ T cells showed a memory type response with rapid γδ T cell expansion after a rechallenge leading to clearance of detectable bacteremia(44). Recently, long term expansion of γδ T cells in CMV-seropositive individuals followed by quicker responses to rechallenge in graft recipients by γδ T cells with an effector/memory phenotype was observed(45). In the

present study mice that had initially undergone WSN-OVA infection showed a strong increase in the number of αβTCR transduced γδ T cells after rechallenge with rVV-OVA. The magnitude and kinetics of the secondary T cell response of the αβTCR transduced γδ T cells were comparable to those observed in the primary response, indicating that αβTCR transduced γδ T cells are able to persist long-term *in vivo* and to mount efficient recall responses.

For successful clinical application of αβTCR engineered γδ T cells some criteria have to be met. Sufficient numbers of γδ T cells have to be available by easy isolation methods, isolated γδ T cells must have the capacity not only to survive but also to proliferate *in vitro* and it must be possible to induce sufficient activation of the isolated γδ T cells to enable retroviral transduction of the αβTCR and the relevant coreceptor. Human γδ T cells can be easily isolated from peripheral blood since percentages of γδ T cells up to 10% of peripheral blood mononuclear cells can be found(46,47). It has been shown possible to expand isolated γδ T cells *in vitro* without deterioration of their effector functions(48). Recently, two studies demonstrated the feasibility of clinical application of adoptive immunotherapy of unmodified γδ T cells(49,50). Up to 8 x 10^9 infused *in vitro* cultured γδ T cells were well tolerated by patients and *in vivo* anti-tumor reactivity was observed. The feasibility of αβTCR and coreceptor transfer to γδ T cells was demonstrated in our previous study in which αβTCR engineered γδ T cells were found to proliferate vigorously *in vitro*, to produce cytokines in an antigen specific manner and to exert antigen specific cytotoxicity against leukemic cells(24). These data together with the data from the present study provide evidence that γδ T cells equipped with an αβTCR can function specifically

both *in vitro* and *in vivo* and that these effector cells may offer a safe alternative for the use of αβ T cells in immunotherapy(19).

In summary, we here show that αβTCR transduced γδ T cells proliferate *in vivo* and produce cytokines in an antigen-dependent fashion. In addition, these gene-modified γδ T cells persisted *in vivo* and were capable of mounting efficient recall responses. The combination of functional reactivity and lack of mixed dimer formation makes αβTCR modified γδ T cells an attractive cell population for future clinical studies.

Acknowledgments

The authors thank Reinier van der Linden, Menno A.W.G. van der Hoorn and Maarten van de Keur for expert technical assistance.

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Figure legends

Figure 1. Antigen specific expansion and cytokine production of γδ T cells transduced with an αβTCR. Purified γδ T cells from Ly5.2$^+$ mice were transduced with the OT-I TCR in combination with CD8αβ and infused intravenously into Ly5.1$^+$ mice. Subsequently, the mice received an intranasal infection with either WSN-OVA or influenza A/HK/2/68. **A.** Representative FACS profiles at day 9 gated on CD8$^+$ cells from mice that received OT-I + CD8 transduced γδ T cells intravenously and which were subsequently infected with either influenza A/HK/2/68 (*upper panels*) (n=4) or WSN-OVA (*middle panels*) (n=4). In addition, flow cytometry profiles at day 9 are shown gated on Ly5.2$^+$ CD8$^+$ cells of the WSN-OVA infected mice (*lower panel*). **B.** Representative flow cytometry profiles of intracellular IFNγ staining of peripheral blood derived lymphocytes from Ly5.1$^+$ mice at the peak of the response (day 9) that received Ly5.2$^+$ OT-I + CD8 transduced γδ T cells, and were subsequently infected with either influenza A/HK/2/68 (data not shown) (n=4) or WSN-OVA (n=4). Peripheral blood derived lymphocytes were *ex vivo* stimulated with the $OVA_{257-264}$ peptide or SV40 large $T_{404-411}$ control peptide, and subsequently, antigen specific intracellular IFNγ production was determined by flow cytometry. **C.** Representative flow cytometry profiles of intracellular IFNγ staining of total splenocytes derived from Ly5.1$^+$ mice at the peak of the response (day 9) that received Ly5.2$^+$ OT-I + CD8 transduced γδ T cells, and were subsequently infected with either influenza A/HK/2/68 (n=4) or WSN-OVA (n=4). Splenocytes were *in vitro* stimulated with SV40 control peptide (data not shown) or OVA peptide, cultured for 14 days, restimulated with OVA or SV40 control peptide and subsequently, antigen specific intracellular IFNγ production was determined by flow cytometry.

αβTCR transduced γδ T cells proliferated *in vivo* and produced cytokines upon antigen specific stimulation.

Figure 2. Self-antigen specific reactivity of αβTCR transduced γδ T cells.

Purified γδ T cells were transduced with the OT-I TCR in combination with CD8αβ and infused intravenously into RIP-OVA^{HI} mice which were subsequently infected intranasally with either influenza A/HK/2/68 (n=3) or WSN-OVA(n=3), or were infected intraperitoneally with rVV-OVA (n=4). The percentages of Vα2^{+} TCRγδ^{+} cells of total CD8^{+} cells in peripheral blood at different time points after infection are shown. Data represent group averages ± SD. OT-I TCR transduced γδ T cells expanded *in vivo* in an antigen specific manner in the absence of an endogenous OVA-specific T cell response.

Figure 3. Analysis of intestinal sites for the presence of TCR-transduced γδ T cells. RIP-OVA^{HI} mice received an adoptive transfer of purified γδ T cells transduced with CD8αβ (mock) (n=3), γδ T cells transduced with the OT-I TCR + CD8αβ (n=3), or no cells (n=3) and were subsequently infected with WSN-OVA. **A.** The percentages of TCR Vα2^{+} TCRγδ^{+} cells of total CD8β^{+} cells in peripheral blood at different time points after infection are shown. Data represent group averages ± SD. **B.** At the peak of the response (day 12) cells derived from blood, lamina propria (LP) and intra epithelial lymphocyte (IEL) fractions were analyzed by flow cytometry. The total number of γδ T cells in blood, IEL and LP fractions varied from approximately 5% to 36%, respectively (representative histograms of OT-I TCR + CD8αβ group shown). **C.** Representative flow cytometry profiles are shown of gated TCRγδ^{+} cells

from blood, IEL and LP fractions of RIP-OVA^{HI} mice that received γδ T cells transduced with CD8αβ (mock), γδ T cells transduced with the OT-I TCR + CD8αβ, or no cells and were subsequently infected with WSN-OVA. OT-I TCR + CD8αβ transduced γδ T cells were found mainly in peripheral blood and no αβTCR transduced γδ T cells were found in the intestinal epithelial sites.

Figure 4. *In vivo* persistence of αβTCR transduced γδ T cells. RIP-OVA^{HI} mice received an intravenous infusion of OT-I CD8 transduced γδ T cells and were subsequently infected with influenza A/HK/2/68 (n=3) (closed circles) or WSN-OVA (n=3) (open triangles). At various time points the percentage of OT-I^{+} CD8^{+} γδ T cells in blood was analyzed. 12 weeks after the primary response the mice that initially received a WSN-OVA infection (n=3) were challenged with an intraperitoneal injection of rVV-OVA. Data represent group averages ± SD. αβTCR transduced γδ T cells persisted *in vivo* and generated T cell memory.

Figure 5. Cytolytic activity of αβTCR transduced γδ T cells. RIP-OVA^{HI} mice received an intravenous infusion of OT-I CD8 transduced γδ T cells and were subsequently infected with influenza A/HK/2/68 (n=1) or WSN-OVA (n=3). CFSE^{low} labeled unpulsed splenocytes and CFSE^{high} labeled OVA peptide pulsed splenocytes were injected at a 1:1 ratio around the peak (day 11) of the primary response. Flow cytometric analysis of spleen cells of influenza A/HK/2/68 (*upper panel*) or WSN-OVA (*lower panel*) was performed 5h after transfer of CFSE-labeled cells. OT-I CD8 transduced γδ T cells were capable of antigen specific cytolytic activity *in vivo*.

Figure 1

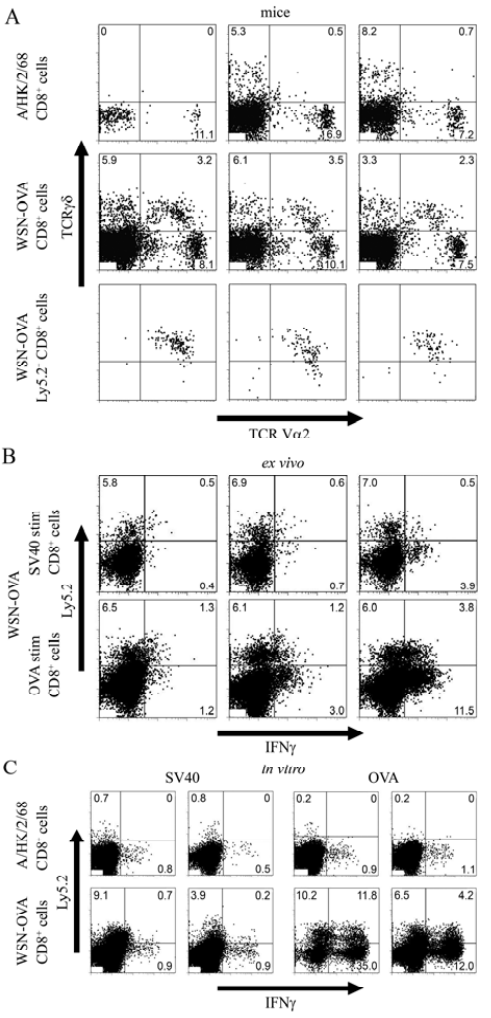


Figure 2

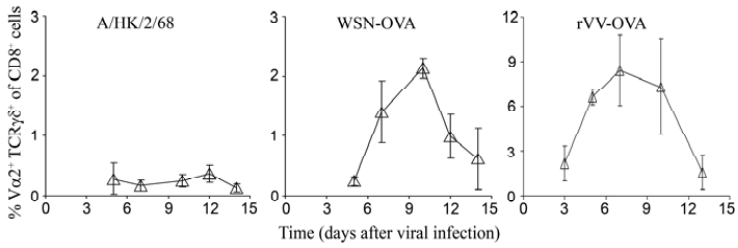


Figure 3

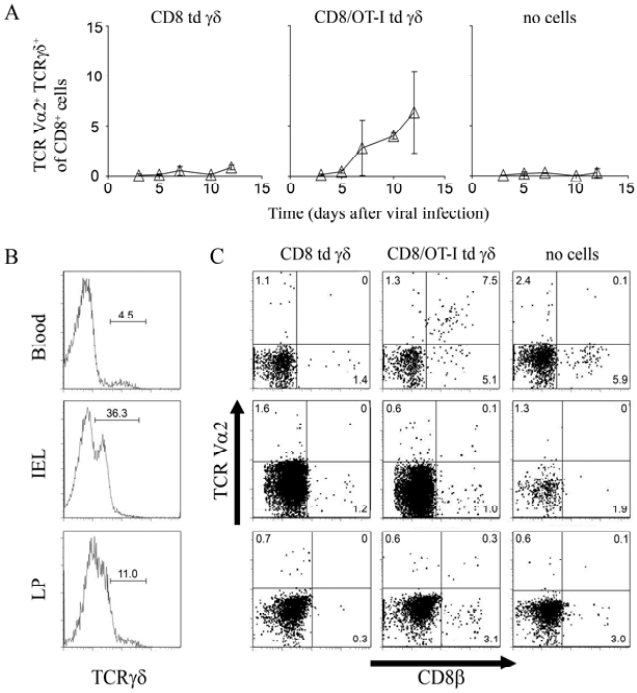


Figure 4

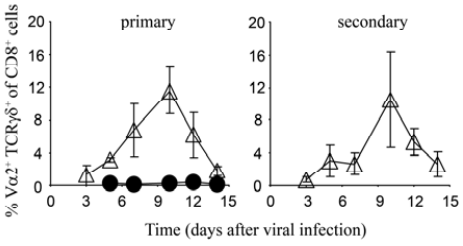
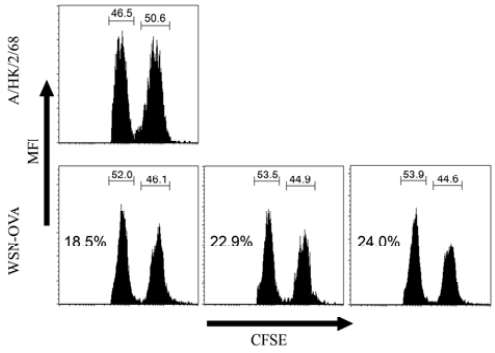


Figure 5


46.5
50.6
A/HK/2/68
MFI
52.0
46.1
53.5
44.9
53.9
44.6
WSN-OVA
18.5%
22.9%
24.0%
CFSE

Chapter 6

Summary and Discussion

Summary and discussion

The human body is equipped with a large array of defense mechanisms that all work together in preventing foreign pathogens from entering. Important components within the immune system are the cytotoxic T cells. Cytotoxic T cells are constantly scanning the body for invaders. They scan all cells they encounter on their way for signs of infection. This scanning is mediated through the T cell receptor (TCR). Every T cell has its own unique T cell receptor, creating a large diversity within the T cell population. This enables the immune system to be prepared to fight the many different pathogens it can be confronted with.

Virtually all cells in the body constantly express protein fragments from proteins synthesized within the cell on their cell surface bound to MHC class I molecules, making the cell surface a reflection of what is going on inside a cell. MHC class I molecules are anchored in the cellular membrane and are highly suitable to bind small protein fragments, antigens, and to present them to T cells. Triggering of the TCR by binding of an antigen loaded MHC complex sends an activation signal towards the T cell nucleus, causing the T cell to divide. This way, an army of antigen specific cytotoxic T cells comes into being, all directed towards the same antigen. Infected cells will be killed and the infection will be halted. After T cells have undergone such massive proliferation upon antigenic stimulation, most of the cells die off. However a small portion of the antigen specific T cells remains and becomes memory T cells. Memory T cells are, upon secondary encounter with their cognate antigen, able to respond quicker and are present in higher numbers than naive T cells specific for the same antigen[1]. Furthermore, memory T cells have an increased capacity to patrol peripheral organs. This way the immune system is prepared upon re-entry of pathogens in the body.

Identification of specific T cells

As mentioned above, cells present a collection of antigens on the cell surface bound to MHC class I molecules and antigen specific T cells are defined by their reactivity with a specific antigen-MHC complex. Because of this specificity, soluble MHC molecules that contain an antigen of interest can be used as a tool to detect antigen specific T cells[2]. To

this purpose, MHC molecules loaded with a given antigen first need to be produced in a multivalent format, as the strength of a single pMHC-TCR is insufficient. Soluble MHC molecules can be created that contain a biotin tag and this biotin tag can bind to fluorescently labeled streptavidin molecules. As each streptavidin molecule can bind four biotin molecules, this results in the formation of so-called MHC tetramers (tetra=four). MHC tetramers can be used to follow antigen specific T cells, e.g. in peripheral blood samples or tissue samples. In addition, there is a growing interest in the use of MHC tetramers to purify antigen specific T cells for adoptive cell therapy. Moreover, tetrameric complexes can be used to identify novel cancer-associated target epitopes for T cells. Human cancer cells are genetically instable, increasing the risk of outgrowth of escape variants when the cells are targeted considerably. Therefore, to effectively treat patients with adoptive T cell therapy or vaccination it is important to identify as many tumor antigens as possible. This emphasizes the need for a fast and efficient method to screen for specific T cell antigens. In **chapter 2** we describe a novel method that can be used to create very large collections of MHC tetramers. Specifically, we describe how MHC molecules loaded with a UV sensitive peptide can be transformed into tetramers of a desired specificity and how we have used this strategy to identify a novel T cell epitope in the nucleoprotein sequence of the H5N1 avian influenza virus. This novel MHC exchange method allows the high throughput analysis of T cell antigens within viral genomes and on tumor cells**.**

Adoptive Immune therapy

Adoptive cell therapy (ACT). The prime function of T cells is to scan for cells that have been infected by a pathogen and to subsequently eliminate them. This specific recognition of infected cells is based on the fact that T cells that recognize MHC molecules complexed with endogenous antigens are normally removed from the T cell repertoire[3]. This ability to recognize 'foreign' but not 'self' limits the capacity of T cells to recognize human cancer cells. Specifically, the majority of human cancers are not virus-induced, and hence do not express the same type of shared foreign antigens as are present in infected cells. In addition, while there is an accumulation of genetic mutations

in human cancers, the vast majority of these mutations is patient-specific, thus hindering the development of T cell-based anti cancer therapies.

Naturally occurring T cell responses that are directed against so-called shared tumor associated antigens (TAAs) are nevertheless found in some patients that carry non-virally induced tumors. These shared TAAs come in two different flavors. First, aberrant expression of non-mutated self-antigens, normally present on specific tissue types, can be observed on cancer cells. Secondly, non-mutated, cell-lineage-specific antigens expressed on tumors can be recognized by specific T cells. In both these cases, T cell reactivity can only be found when self tolerance against these antigens is incomplete and these T cell responses are often weak. In line with this, natural T cell responses against non-mutated self antigens are rarely sufficient for tumor rejection, as has been observed in humans and animals[4,5,6]. The key to this failure of tumor specific T cells may be found in the scarcity of high affinity tumor specific T cells. Evidence for the thought that infusion of small numbers of high affinity T cells may be of value comes from the observed effect of donor lymphocyte infusion in certain hematological malignancies[7]. On the other hand, evidence for the thought that infusion of large numbers of lower affinity T cells against self antigens may be of value comes from the observed effect of ACT with autologous ex vivo expanded T cells in melanoma patients[8-11]. ACT for melanoma comprises infusion of tumor infiltrating lymphocytes (TIL) that have been isolated from tumor tissue of the patient. These cells are expanded in the laboratory to large amounts en subsequently returned to the patient. This way, T cells with the highest *in vitro* tumor reactivity may be selected from the tumor specific T cell pool and the cells are expanded *in vitro* circumventing any possible suppressing activities by the tumor. Furthermore, patients are treated with lymphodepleting chemotherapy prior to adoptive transfer, generating an optimal host environment for the transferred T cells as T cells have been demonstrated to expand massively and acquire an effector phenotype in lymphodepleted hosts. A number of clinical trials has shown promising results using this type of strategy, for instance the treatment of melanoma patients with ex vivo expanded tumor infiltrating lymphocytes[8-11]. As some of the patients treated within these protocols display very marked responses against melanocyte differentiation antigens, these data suggest that T cells specific for non-mutated self antigens can have a meaningful anti-tumor effect. However, it should

be stressed that formal proof for the involvement of the observed MDA-specific T cells in tumor control is lacking and it remains possible that T cell responses against patient-specific neo-antigens do play a significant role.

TCR gene therapy. While these adoptive therapy trials are of great value to define the potential of T cell based immune therapy, adoptive therapy has its drawbacks. First, not in all cancer patients tumor specific T cells can be found, which poses the largest obstacle. Second, the growth of tumor specific cells in the lab is a laborious process for each individual patient and the cells need to be activated with artificial stimuli to large numbers before they can be returned to the patient. Moreover, cells found in a given patient can only be used for adoptive immune therapy in that specific individual because polymorphic differences between donor and recipient prevent allogeneic cell transfer. To circumvent the above mentioned issues, it has been proposed to make use of the sole unique property of tumor specific T cells, namely the tumor specific T cell receptor. Because the TCR is the determining factor in the recognition process between T cell and cancer cell, T cells may be 'redirected' towards an antigen of choice by transfer of TCR genes[12-16]. This way, TCR genes can be used to provide many patients with tumor specific T cells as long as the recipient individual has the correct MHC allele necessary to present the tumor specific antigen to the introduced TCR. The transfer of TCR genes from one cell to another is called 'TCR gene transfer'. It is a fast method to create large amounts of tumor specific T cells and above all is not restricted to a single patient. The research described in this thesis focuses mainly on the tracking of the antigen specific T cell repertoire and the manipulation of this repertoire through TCR gene transfer.

Safety. TCR gene transfer is performed in the laboratory by making use of virus particles to introduce the TCR genes into T cells. This technique is called retroviral transduction of T cells. The transfer of TCR genes by retroviral transduction is not without risk and two main risk categories can be distinguished. First, the integration of the introduced DNA into the host genome is largely random and such integration can disrupt the regulation of neighboring genes. The danger of random insertion of DNA sequences in the host genome was demonstrated earlier in a highly publicized human gene therapy trial in

which common γ-chain cytokine receptor deficient SCID-X1 patients were treated with stem cells transduced with the gene that codes for the common γ-chain[17]. Although this was the first successful gene therapy trial, some of these patients developed T cell leukemia as a consequence of the gene therapy[18]. However, for T cells there is presently no evidence for lymphomagenesis after gene transfer and even though it will be important to develop safer retroviral vectors with either a more controlled manner of inserting DNA content or that disrupt local gene expression to a lesser extent, this appears not to be a critical issue for T cells. A possible explanation for the higher risk of hematopoietic stem cell (HSC) transduction as compared to T cell transduction may be formed by the fact that many genes responsible for self-renewal capacity will be active in HSC but not T cells. As oncoretroviral vectors predominantly integrate in the proximity of active genes, the risk of disregulation of critical oncogenes may be higher in HSC than in T cells. Furthermore, a potential transforming activity of the introduced transgene itself may have contributed to oncogenesis in these patients[19], a feature which is not known to apply to TCR genes.

Second and more specific for TCR gene transfer, there is the risk of unwanted reactivity of the TCR-modified T cells[20]. Three scenarios exist that could lead to such unwanted reactivity as a consequence of TCR gene transfer. First, the T cell receptor consists of two different chains, the alpha and beta chain. These chains form a dimer on the cell surface of T cells. Because T cells used for retroviral transduction have their endogenous TCR in addition to the transferred TCR, it is possible that the introduced chains form heterodimers with the endogenous chains, leading to the formation of new TCRs with unknown specificity and which can therefore potentially be auto reactive. Second, allo-reactivity of the introduced TCR towards MHC molecules previously non-encountered might cause auto immune reactions by the transduced T cells. This includes both direct allo-reactivity towards foreign MHC molecules or towards self antigens presented by foreign MHC molecules. Third, activation of ignorant self reactive T cells via the introduced TCR might trigger auto reactivity after adoptive transfer. The risk of any of these unwanted reactions by transduced T cells was assessed in **chapter 3** by adoptively transferring a large group of partially MHC mismatched mice with T cells transduced with either the D^b-restricted influenza A specific F5 TCR or the K^b-restricted ovalbumin

specific OT-I TCR. Allowing the introduced TCRs to interact with previously unseen MHC molecules could give an indication of the risk of the above mentioned scenarios. The mice were subsequently infected with a virus expressing the cognate antigen to make the T cells expand *in vivo*. Expansion of the transduced cells in peripheral blood was measured and after several months the mice were sacrificed for pathological analysis. This analysis was performed on primary target organs for auto-immune pathology such as intestine, skin, and liver, but also a series of other organs was examined thoroughly. Examination of over 80 mice having received TCR transduced T cells revealed no apparent signs of auto-immunity, indicating that TCR gene therapy can be relatively safe, at least under these conditions and for the two TCRs tested.

Human studies have shown beneficial effects of host-conditioning regimens such as lymphodepletion prior to ACT and high dose IL-2 treatment on the efficacy of ACT, improving the effect of the transferred T cells[21]. Because of this, this type of conditioning has also been used in the one published TCR gene therapy trial. However, a recent study has shown that under lymphodepleting conditions combined with high dose IL-2, unwanted reactivity of TCR transduced T cells can be observed in mice (Bendle et al., pers. comm). In these experiments the formation of mixed dimers is likely to be the cause of the observed self reactivity, as both TCR transgenic and mock-transduced T cells do not cause pathology. Moreover, single alpha- or beta-chain transduced T cells also induce pathology, to a similar extent as two-chain transduced cells. In the past years, it has been shown by several groups that it is possible to reduce mispairing of the introduced TCR chains with endogenous chains by manipulation of the TCR interface of the introduced chains[22-25]. Another strategy to prevent mixed dimer formation after TCR gene transfer is described in **chapter 5**. To avoid the formation of mixed TCR dimers, one can use a specific subset of T cells as host cells for the TCR genes, the gamma delta T cells. This small subset of T cells expresses TCR gamma and delta genes instead of alpha and beta genes. Gamma delta T cells may form very attractive host cells for alpha beta T cell receptors because gamma and delta TCR chains can not dimerize with alpha and beta TCR chains[26,27]. It has previously been shown that human gamma delta T cells that are transduced with an alpha beta TCR can be activated through the introduced TCR[28]. Here we assessed whether mouse gamma delta T cells that serve as host cells for an alpha beta

TCR can be functional in an *in vivo* model. To this purpose we transduced gamma delta T cells with the OT-I alpha beta TCR and measured responsiveness of the cells in peripheral blood of mice. We investigated kinetics of the response and homing to lymphoid organs. Also the in vivo functionality of the cells was characterized. Murine gamma delta T cells turn out to be suitable hosts for a functional alpha beta TCR. They get activated in an antigen-specific manner and produce IFN-γ upon activation. However in comparison to TCR transduced alpha beta cells they do not perform better and do not seem to reach the same level of expansion upon stimulation *in vivo*. The performance of the transduced gamma delta cells *in vivo* may be hampered somewhat by the laborious isolation procedure from splenocytes; their frequency is low and multiple sorting steps are needed to render a pure population. This in contrast to human gamma delta cells which can easily be isolated in large numbers from peripheral blood. In addition, it is possible that the proliferative burst of gamma delta T cells is on average lower than that of alpha beta T cells. If this is the case, this may imply that clinical implementation of gene therapy with TCR-transduced gamma delta T cells may be more difficult. It is noted that further analyses will be required to determine to what extent redirected gamma delta and alpha beta T cells different with respect to aspects such as homing properties and sensitivity to negative regulators of T cell activity such as TGF-beta.

Now it is becoming clear that mixed TCR dimers can cause serious side-effects under certain conditions it will become critical to test the value of these engineering approaches for preventing this pathology. Along the same line, so-called 'suicide switches' have been generated which can be utilized to force TCR transduced cells into apoptosis once they are *in vivo*[29,30] and it may be valuable to test their potential in halting mixed dimer induced pathology. In summary, although the first clinical trial using TCR transduced T cells has not revealed unacceptable toxicity[31], additional manipulation of either the TCR or of the T cells that are being modified may add to the acceptability of TCR gene therapy for clinical purposes in the future.

Circumventing tolerance. Another question to be answered was whether it is possible to break T cell tolerance towards a defined self-antigen by TCR gene transfer. T cells directed against a self-antigen might encounter regulatory mechanisms once transferred

into the recipient, such as regulatory T cells or peripheral tolerization. Up to this point all TCR gene transfer studies were performed in a viral infection model, using a TCR directed against a foreign antigen. Viral models are however less relevant for TCR gene therapy as an anti-cancer treatment to be applicable for the targeting of defined tumor-associated self-antigens. For this purpose, T cells from RIP-OVA mice, which express ovalbumin as a self-antigen under control of a beta cell-specific promotor and are hence tolerant towards ovalbumin, were transduced with the ovalbumin specific OT-I TCR and transferred back into RIP-OVA hosts as described in **chapter 3**. Destruction of the ovalbumin expressing beta cells led to diabetes induction in these mice, indicating killing activity of the reprogrammed T cells towards cells expressing a defined self antigen, in this case pancreatic beta cells. More recently we have shown that also in animals that spontaneously develop prostate carcinomas it is possible to break tolerance through the adoptive transfer of TCR transduced tumor-specific T cells[32]. In these experiments, tumor bearing mice were treated with T cells transduced with a TCR specific for an oncogene expressed by the tumor for which the animals are normally tolerant. Post-therapy analysis of the animals showed a marked reduction in tumor progression in mice that had received TCR gene therapy in contrast to mice that had received mock treatment. These data show that it is possible to reprogram T cells to target tumors that strictly express self-antigens.

***In vivo* persistence.** Long term persistence and memory development of transduced T cells might prove valuable in the therapeutic treatment of cancer patients. First, it has been shown in human ACT clinical trials that the persistence of adoptively transferred T cells *in vivo* is correlated with improved clinical outcome[33,34]. Second, in case TCR gene therapy will be considered in the more distant future as an adjuvant treatment for patients that are at risk of relapse, the long term presence of the gene-modified . In **chapter 4** it is described what happens with TCR transduced T cells over prolonged periods of time. We show that transduced T cells persist *in vivo* for long periods, up to six months, without antigenic stimulation indicating that the infused cells are not immunogenic after gene transfer. Upon antigenic restimulation *in vivo* however, we detect only marginal secondary responses of TCR transduced T cells when using the same virus as for the primary response. Secondary stimulation with a different virus strain containing the

cognate antigen does however lead to expansion, indicating that there is no intrinsic lack of capacity to form T cell memory. It is noted that this secondary expansion is not faster or more pronounced than that of the primary response as would be expected for classical memory T cell responses. We therefore assessed what the underlying mechanism for this apparent impairment in memory formation might be. We therefore compared TCR transduced T cells and TCR transgenic T cells specific for the same epitope, the latter either used as naive T cells or after having undergone a 'mock retroviral transduction' From this comparison we can conclude that TCR transduced T cells do not differ from TCR transgenic T cells in their capacity to mount secondary responses. Because of this, we consider that the difference in secondary response between the TCR transduced T cells and the 'classical'memory response is explained by the fact that the TCR transduced cells that are adoptively transferred already form a large T cell pool available for antigen recognition, resulting in massive expansion upon viral inoculation. The secondary response therefore remains similar in size and kinetics. In summary, the longevity of the infused T cells observed in these experiments (up to 18 months) gives an indication that these gene-modified T cells are not immunogenic, at least in this model system, and the possibility to re-activate them after prolonged periods indicates that there is no (irreversible) silencing of the genes introduced, or a defect in memory T cell formation.

Conclusion. Recently the field of adoptive immune therapy has advanced with the first human trial with TCR transduced T cells in melanoma patients[31]. This was the first time that TCR transduced T cells were clinically administered to humans and the results hold promise for this form of immunotherapy. However, there is still room for improvement as the response rate within this clinical trial was significantly lower than in the TIL-trials performed by the same group. T cell avidity may be reduced in TCR transduced T cells due to mispairing of the introduced chains, leading to reduced surface expression of the introduced TCR and the affinity of the TCR used in this first trial was relatively low. The strategies that prevent TCR mispairing can in part solve this problem. There are more strategies however to improve TCR surface expression and TCR affinity. Surface expression can be improved by the strategies mentioned earlier but also by optimizing the retroviral vector used to transfer the TCR genes into T cells[35,36] or by codon-optimization

of the TCR genes[37,38] and TCR signalling may be potentially improved by modification of intracellular signalling domains. The best strategy will be different for each individual TCR, depending on the intrinsic properties of the TCR. So-called strong TCRs may functionally improve by codon-optimization, whereas weak TCRs may benefit more from increased stability and expression level[39,40]. Independent of the TCR format used, the conditions under which the T cells are cultured prior to infusion and in particular the cytokines used may greatly influence their efficacy in vivo. Standard *in vitro* culture protocols describe IL-2 for expanding T cells, however IL-2 may not be the optimal cytokine to prepare the most optimal cells for *in vivo* use[41]. Specifically, culture in IL-2 leads to a more differentiated phenotype of the T cells, while retention of a more naive phenotype of the cells is associated with superior *in vivo* activity[42].

This thesis is mainly focused on research into a new strategy to treat cancer patients with adoptive immune therapy, TCR gene therapy. Data from mouse models and clinical trials clearly indicate that TCR gene therapy may prove a powerful tool to generate unique and tumor-specific T cells. TCR gene transfer enables the targeting of self-antigens that is not possible within the endogenous immune system and thus epitomizes a unique tool in cancer therapy. However, the current data also indicate that the efficacy of TCR transduced T cells needs to be improved in order to obtain better results. The above mentioned strategies to improve surface expression and affinity of the introduced TCR may prove valuable tools for this purpose. But, as it appears now, the safety of TCR gene therapy will require equal attention for it to become a genuine success. As has been shown, host conditioning is a major contributing factor in the success of ACT-trials and in murine models[43], but may also be the major contributing factor in the track leading to side effects. Depletion of endogenous regulatory mechanisms and stimulation of the innate immune system may give the infused cells the opportunity to derail. If the newly developed strategies for the improvement of safety and efficacy of TCR gene therapy can successfully be implemented into a clinical protocol the future for TCR gene therapy may be bright, either as part of therapy in combination with other anti-cancer drugs or even as a separate treatment. Recently it was published in the Dutch media that cancer will become the primary cause of death within a few years in the Netherlands, taking over this

questionable honor from coronary disease. Because cancer therapy is still often palliative instead of curative, the search for new ways of treatment is essential and hopefully TCR gene therapy can become one of those.

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Nederlandse samenvatting

List of publications

Curriculum Vitae

Samenvatting

Ons lichaam is uitgerust met een uitgebreid arsenaal aan afweercomponenten die samen er voor zorgen dat pathogenen van buitenaf geen kans maken zich in ons lichaam te verspreiden. Een belangrijke factor in ons afweersysteem zijn de zogenaamde T cellen. T cellen zijn een onderdeel van het adaptieve immuunsysteem, dat wil zeggen; ze zijn in staat te reageren op tot dan toe onbekende pathogenen en zich aan te passen aan het specifieke pathogeen om het vervolgens uit te schakelen. De T cellen kunnen worden onderverdeeld in $CD4^+$ T helper cellen en $CD8^+$ cytotoxische T cellen. Deze twee celtypen werken nauw samen in de immuunrespons. Cytotoxische T cellen zijn voortdurend bezig te speuren naar indringers. Ze scannen cellen die ze onderweg tegenkomen op signalen van infectie met een pathogeen. Dit scannen gebeurt via eiwitreceptoren op het celoppervlak van de T cel, de T cel receptor (TCR). Elke T cel heeft zijn eigen, unieke T cel receptor wat een grote diversiteit van T cellen oplevert. Hierdoor is het lichaam in staat voorbereid te zijn op de vele verschillend pathogenen waarmee het geconfronteerd kan worden.

Alle cellen in het lichaam brengen voortdurend fragmenten van eiwitten uit hun binnenste tot expressie op hun celoppervlak gebonden aan MHC moleculen waardoor het oppervlak van de cel een weergave is van wat er binnenin gaande is. Deze eiwitfragmenten noemen we epitopen of antigenen. Zodra T cellen een lichaamsvreemd eiwit herkennen via de T cel receptor komen ze direct in actie. Door het activeren van de T cel receptor wordt de bewuste T cel geprogrammeerd tot delen. Hierdoor ontstaat een leger aan T cellen met dezelfde T cel receptor en dus dezelfde specificiteit voor een bepaald antigeen. T cellen bezitten nog een belangrijke eigenschap die het immuunsysteem in staat stelt adequaat te reageren op indringers van buitenaf. Nadat T cellen een specifieke afweer reactie hebben doorgemaakt sterft een groot deel van de cellen af. Echter een kleine fractie blijft in leven en ontwikkelt zich tot memory cellen. Deze cellen blijven circuleren en zijn bij een volgende ontmoeting met hun specifieke antigeen in staat sneller en heftiger te reageren dan de eerste keer. Hierdoor wordt een eventuele terugkeer van een bepaald pathogeen voorkomen. Zodoende worden geïnfecteerde cellen opgespoord, geëlimineerd en de infectie tot staan gebracht.

T cellen zijn dus uitgerust om zieke cellen op te sporen en ze te vernietigen. Echter, soms is een cel ziek en wordt hij toch niet herkend door het immuunsysteem. Dit is vaak het geval bij het ontstaan van kanker. Dit gebeurt omdat kankercellen wel abnormaal zijn, maar zij brengen veelal eiwitten aan het oppervlak die lichaamseigen zijn en worden door het immuunsysteem niet als ziek herkend. Hierdoor krijgen kankercellen vaak de kans ongestoord te delen en te migreren. In sommige gevallen kan de kanker wel herkend worden door het immuunsysteem. Dit kan gebeuren als er mutaties optreden in de lichaamseigen eiwitten van de kankercel of wanneer de kanker ontstaan is door virale infectie. Virale eiwitten op het oppervlak van kankercellen en in mindere mate gemuteerde lichaamseigen eiwitten woerden wel door het immuunsysteem herkend. De reden dat ook in zulke gevallen de kanker ongestoord kan groeien, kan aan de affiniteit van de T cellen liggen, met andere woorden, de mate waarin T cellen hun specifieke antigeen herkennen. Als de affiniteit van T cellen laag is, zullen kankercellen makkelijker ontkomen aan eliminatie. Tevens kunnen kankercellen gebruik maken van ontsnappingsmechanieken. Ze kunnen bijvoorbeeld het specifieke antigen waar de T cellen op af gaan dusdanig reguleren dat het niet meer tot expressie komt en de kankercel niet meer als vreemd herkend word of stoffen uitscheiden die T cellen remmen in hun functie.

T cellen die kankercellen kunnen herkennen door kleine veranderingen, hetzij in de lichaamseigen eiwitten, hetzij door virale eiwitten noemen we tumorspecifieke T cellen. Deze tumorspecifieke cellen worden gevonden in kankerpatiënten, echter vanwege bovengenoemde problemen hebben deze cellen meestal geen genezend effect.

Het feit dat in patiënten tumorspecifieke T cellen worden gevonden is een opstap voor de zogenaamde adoptieve immuuntherapie. Adoptieve therapie bestaat meestal uit tumorspecifieke T cellen die worden ontdekt in een patiënt. Deze cellen worden dat geïsoleerd uit de patiënt en in het laboratorium opgekweekt tot grote hoeveelheden waarna ze worden teruggegeven aan diezelfde patiënt. Nu de cellen in getale zijn toegenomen worden ze in staat gesteld de tumor te lijf te gaan. Uit een aantal klinische onderzoeken is gebleken dat adoptieve therapie een bijdrage kan leveren aan de behandeling van kanker.

Nu zitten er een aantal nadelen aan adoptieve immuuntherapie. Ten eerste kunnen niet in elke willekeurige kankerpatiënt tumorspecifieke T cellen worden gevonden, wat het grootste obstakel is. En ten tweede kost het opgroeien van deze cellen in het lab veel tijd en moeite per patiënt. De cellen moeten met kunstmatige stimuli tot delen worden aangezet en tot grote aantallen worden vermeerderd. Bovendien kunnen de tumorspecifieke T cellen van een specifieke patiënt alleen voor diezelfde patiënt worden gebruikt vanwege afstotingsmechanismen.
Om bovengenoemde problemen te omzeilen, kan men er ook voor kiezen gebruik te maken van de unieke eigenschappen van tumorspecifieke T cellen, namelijk de tumorspecifieke T cel receptor. Want de T cel receptor is de bepalende factor in het proces van T cel- kankercel interactie. Aangezien het mogelijk is T cel receptor genen van een tumorspecifieke T cel te isoleren en vervolgens over te zetten in een andere T cel, kan men door tumorspecifieke T cel receptoren te isoleren uit patiënten en die over te brengen naar T cellen van een ander individu nieuwe tumorspecifieke T cellen maken voor dat individu. Dit is een snelle manier om grote hoeveelheden tumorspecifieke T cellen te verkrijgen en is bovendien niet patiëntgebonden omdat de exogene TCR genen worden ingebracht in de eigen T cellen van de patiënt.

Zoals eerder gezegd presenteren cellen hun antigenen op het celoppervlak gebonden aan MHC moleculen. Deze MHC moleculen zijn verankerd in de celmembraan en zijn speciaal uitgerust om antigenen aan de T cel receptor te presenteren. De TCR herkent dan ook uitsluitend epitopen als die op een MHC molecuul gepresenteerd worden. Deze MHC moleculen kunnen ook gebruikt worden om specifieke T cellen te detecteren, bijvoorbeeld in perifeer bloed. Losse MHC moleculen met een biotine molecuul eraan kunnen worden beladen met specifieke epitopen naar keuze en door middel van een fluorescent gelabeld streptavidine molecuul aan elkaar gelinkt worden. Zo ontstaan MHC tetrameren, die door het fluorescente label zichtbaar gemaakt kunnen worden in de flow cytometer. Omdat deze tetrameren beladen zijn met het antigeen van interesse zullen alleen T cellen met de TCR met de juiste specificiteit eraan binden. Zo kan men specifieke T cellen in bijvoorbeeld perifeer bloed volgen en kwantificeren. MHC tetrameren zijn een belangrijke ontdekking in de immunologie geweest omdat sindsdien

het zeer gemakkelijk is specifieke T cel responsen te volgen terwijl dit voorheen nauwelijks mogelijk was.
In **hoofdstuk 2** van dit proefschrift wordt een nieuwe, snelle methode beschreven voor het maken van MHC tetrameren die het mogelijk maakt massa productie uit te voeren van veel verschillende tetrameren tegelijk. In hoofdstuk 2 wordt deze methode toegepast om binnen het vogelgriepvirus nieuwe epitopen te vinden die door T cellen herkend kunnen worden. Er wordt beschreven hoe MHC moleculen beladen met een UV gevoelig peptide kunnen worden omgezet in tetrameren van de gewenste specificiteit en hoe een nieuw epitoop is geïdentificeerd in het nucleoproteine van het H5N1 vogelgriepvirus. Deze methode maakt het tevens mogelijk nieuwe T cel epitopen te identificeren van bijvoorbeeld nieuw ontdekte virussen, die weer gebruikt kunnen worden voor de ontwikkeling van vaccins. Bovendien zou deze strategie gebruikt kunnen worden om nieuwe tumorantigenen te identificeren waartegen specifieke T cellen gevormd kunnen worden.

De overdracht van TCR genen naar keuze naar een T cel noemen we ‘TCR gen transfer’. Deze gen transfer vindt plaats in het laboratorium door middel van viruspartikels. De viruspartikels zijn nodig om het DNA de cel in te brengen. We noemen deze techniek retrovirale transductie. De functionaliteit en veiligheid van T cellen die zijn uitgerust met een nieuwe TCR wordt beschreven in **hoofdstuk 3**. Hierin wordt beschreven hoe T cellen met een nieuwe TCR zich gedragen in de muis en dat ze dusdanig geprogrammeerd kunnen worden dat ze lichaamseigen cellen kunnen aanvallen. Ten eerste is onderzocht hoe veilig de nieuwe T cellen zijn in de muis. Het overbrengen van TCR genen in een T cel is niet geheel zonder risico. Deze cellen zijn behandeld met retrovirus om de nieuwe TCR er in te brengen en dragen nu dus ook virale eiwitten met zich mee. Tevens bestaat een TCR uit twee ketens, de alfa en de beta keten. Deze twee verschillende ketens vormen een dimeer op het celoppervlak. Omdat T cellen al een eigen TCR hebben, kunnen de nieuw geïntroduceerde TCR ketens onderling combineren met de endogene TCR ketens. Dit kan leiden tot nieuwe TCRs waarvan men de specificiteit niet kent en deze nieuwe TCRs zouden potentieel gevaarlijk kunnen zijn. Zo zouden ze bijvoorbeeld tot ongewenste auto immuniteit kunnen leiden. In hoofdstuk 3 onderzoeken we in een

grote groep muizen van verschillende genetische achtergrond of er sprake is van auto-immuniteit nadat ze getransduceerde cellen hebben gehad. We kijken enkele maanden na de adoptieve transfer van de T cellen in diverse organen en er blijkt geen aanwijzing te zijn voor auto-immuniteit in deze muizen. Tevens wordt de functionaliteit van de T cellen getest. Ze expanderen na virale infectie van de muizen en bovendien zijn ze in staat om lichaamseigen cellen aan te vallen als ze daartoe geprogrammeerd worden.

In **hoofdstuk 4** word beschreven wat er op lange termijn gebeurt met T cellen nadat ze een nieuwe TCR hebben gekregen. Het overdragen van genen met behulp van retrovirussen houdt in dat de cellen geactiveerd moeten worden, anders nemen ze de viruspartikels niet op. Deze activatie gebeurt in een kweekschaaltje en met een kunstmatige activator, concavaline A. Dit is een onnatuurlijke activatie die ze normaal niet ondergaan en het is daarom belangrijk uit te zoeken of er iets verandert de manier waarop de cellen secundaire responsen doormaken als ze eenmaal in vivo zijn. Met name voor de eventuele behandeling van perifere uitzaaiingen van kanker is het van belang dat de met een nieuwe TCR uitgeruste T cellen langdurig in het lichaam aanwezig blijven en in staat zijn opnieuw te reageren als ze hun specifieke epitoop tegen komen. Tevens heeft onderzoek in klinische trials laten zien dat het langdurig aanwezig blijven van de cellen na adoptieve transfer in de patiënt een gunstig effect heeft op de uitkomst van de behandeling. Wij laten in dit hoofdstuk zien dat genetisch gemodificeerde T cellen een lange levensduur in de muis hebben en ook in staat zijn secundaire responsen te bewerkstelligen indien ze opnieuw met hun antigen in aanraking komen. Ook is er geen verschil tussen onze genetisch gemodificeerde cellen en cellen die van nature al dezelfde TCR hebben. Dit geeft aan dat retrovirale transductie geen nadelig effect op de cellen heeft. Tevens laten we zien dat er in ons model geen clonale expansie van genetisch gemodificeerde cellen optreed, dat wil zeggen dat er geen leukemische transformatie is waargenomen in de muizen.

Het in hoofdstuk 3 beschreven risico dat nieuwe onbekende TCRs worden gevormd kunnen worden na TCR gen transfer kan een nadelig effect hebben op de acceptatie van getransduceerde T cellen als antikanker therapie in de kliniek. Dit risico kan worden voorkomen door zogenaamde gamma-delta T cellen te gebruiken als target voor gen transfer, zoals beschreven wordt in **hoofdstuk 5**. Deze kleine subset van T cellen heeft

een TCR die bestaat uit een gamma en een delta keten. De TCR die gebruikt wordt voor gen transfer is een alfa-beta TCR en recombinatie met gamma-delta ketens is niet mogelijk. Hierdoor wordt het risico van ongewenste TCRs uitgesloten wat van belang is om TCR gen transfer zo veilig mogelijk te maken. Wij tonen aan dat gamma-delta T cellen kunnen dienen als acceptor cellen voor een alfa-beta TCR en dat ze in de muis kunnen expanderen na virus infectie. Naast expansie produceren de getransduceerde cellen ook cytokinen die aantonen dat ze daadwerkelijk functioneel zijn. Verder wordt er in dit onderzoek gekeken naar de locatie van de cellen in de muis na virale infectie. Het is belangrijk te weten waar de cellen heengaan nadat ze geactiveerd zijn. Wij tonen aan dat de cellen naar de primaire plek van infectie, namelijk de longen gaan, en dat ze terug te vinden zijn in secundaire lymfoide organen zoals de lymfklieren van de longen. De gamma-delta cellen zijn niet terug te vinden in het darm epitheel waar normaliter veel van deze cellen te vinden zijn, wat in dit geval positief is aangezien de cellen expanderen na een virale infectie in de longen en dus niet naar het darm epitheel geacht worden te gaan. Gamma-delta T cellen zouden een aanvulling kunnen zijn voor adoptieve therapie omdat ze de veiligheid van deze vorm van kanker immuuntherapie zouden kunnen vergroten, echter wij tonen ook aan dat ze in ons model niet zo krachtig reageren als alfa-beta T cellen.

Recentelijk is er voor het eerst een klinische trial uitgevoerd met melanoma patiënten die TCR getransduceerde cellen hebben gekregen. Deze trial was middelmatig succesvol vergeleken met andere adoptieve immuuntherapie trials. Het is gebleken dat er nog behoorlijk ruimte voor verbetering is op verschillende niveaus, zoals affiniteit van de TCR en de expressie niveau op de celmembraan van de TCR. Tevens zal het onderzoek naar de veiligheid van genetisch gemodificeerde T cellen onverminderd een hoofdpunt blijven alvorens het op grote schaal toegepast kan worden.
Enige tijd geleden werd in de media bekend dat kanker binnen een aantal jaar doodsoorzaak nummer een zal worden, boven hart-en vaatziekten. Omdat kanker een ziekte is met vele variaties en verschijningsvormen is en blijft onderzoek naar nieuwe behandelmethoden onontbeerlijk. Dit proefschrift handelt voornamelijk over de mogelijkheden van adoptieve immuuntherapie met TCR getransduceerde T cellen als

wapen in de strijd tegen kanker. De beschreven data geven de hoop dat TCR gen therapie op termijn mogelijk zal kunnen bijdragen aan de behandeling van kanker en wellicht zelfs een genezend vermogen zal hebben.

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Curriculum Vitae:

Miriam Coccoris werd geboren op 13 juli 1977 te Haarlem. In 1994 behaalde zij haar H.A.V.O diploma op het Kennemer Lyceum te Overveen. In 1996 behaalde zij haar VWO diploma, eveneens op het op het Kennemer Lyceum te Overveen. In dat zelfde jaar begon zij aan de studie Medische Biologie aan de faculteit der Natuurwetenschappen, Wiskunde en Informatica van de Universiteit van Amsterdam. Het propedeutisch examen werd in januari 1998 behaald. Tijdens de studie werden twee stages gelopen. De eerste stage vond plaats op het Specieel Klinisch Immunologisch Lab aan het Academisch Medisch Centrum te Amsterdam onder leiding van Prof. dr. Ineke ten Berge.
De tweede stage werd gelopen op het Nederlands Kanker Instituut op de afdeling Immunologie onder leiding van Prof. dr. Ton Schumacher. In oktober 2000 werd het doctoraal examen behaald. Inmiddels was zij begonnen aan het in dit proefschrift beschreven promotie onderzoek onder supervisie van Prof. dr. Ton Schumacher op de afdeling Immunologie van het Nederlands Kanker Instituut. Sinds februari 2006 is zij werkzaam als postdoc op de afdeling Medische Tumor Immunologie aan het Erasmus Medisch Centrum te Rotterdam.