

Rebalancing Immune Specificity and Function in Cancer by T-Cell Receptor Gene Therapy

Akshata Udyavar · Terrence L. Geiger

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Abstract Adoptive immunotherapy with tumor-specific T lymphocytes has demonstrated clinical benefit in some cancers, particularly melanoma. Yet isolating and expanding tumor-specific cells from patients is challenging and there is limited ability to control T-cell affinity and response characteristics. T-cell receptor (TCR) gene therapy, in which T lymphocytes for immunotherapy are redirected using an introduced rearranged TCR, has emerged as an important alternative. Successful TCR gene therapy requires consideration of a number of issues, including TCR specificity and affinity, optimal gene therapy constructs, types of T cells administered, and the survival and activity of the modified cells. In this review we highlight the rationale for and experience with TCR gene therapy as well as new approaches to enhancing it.

Keywords T-cell receptor · Adoptive immunotherapy · Cancer · Gene therapy

Introduction

Hanahan and Weinberg have defined cancer by six hallmarks: self-sufficient growth, insensitivity to growth inhibition, programmed cell death avoidance, replicative potential, angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). A seventh hallmark is potentially the ability to evade the immune response. Cancer may be kept in check by the immune system and

immune escape is necessary for tumor growth. For the past several decades, efforts to harness the immune system to reject tumors have met with mixed success, and immune modulation as a cancer therapy remains firmly in the experimental realm. Nevertheless, there have been encouraging recent developments in the field, particularly in the ex vivo generation of immunologic effector cells capable of targeting tumors. In this review we highlight a specifically promising therapeutic tool: the use of T lymphocytes genetically modified with tumor-specific T-cell receptors (TCRs).

The Tumor-Specific Immune Response

Whereas the immune system's role in infectious and autoimmune diseases is readily visible, its ability to restrain tumor growth has been less clear. Rare cases of immune-mediated spontaneous tumor regression have been documented (Avril et al. 1992; Halliday et al. 1995). More generally, an effective antitumor response will be an undetectable event marked only by the absence of cancer or delayed tumor growth, making the role of the immune response in suppressing cancer difficult to ascertain. Several lines of evidence, however, indicate that tumors are indeed recognized by the immune system and that immune evasion is an important and sometimes limiting factor in tumor development.

Some cancers are more common in the setting of immune suppression, indicating a potential role for immunosurveillance in preventing tumor growth (Shankaran et al. 2001). Indeed, an adaptive immune response is readily detectable against tumors serologically (Preuss et al. 2002). Furthermore, many types of tumors are infiltrated by significant populations of tumor-specific lymphocytes. In

A. Udyavar · T. L. Geiger (✉)
Department of Pathology, St. Jude Children's Research Hospital,
262 St. Jude Place, D-4047, Memphis TN38105, USA
e-mail: terrence.geiger@stjude.org

models of de novo tumor development, cancers evolve in synchrony with an adaptive antitumor immune response, a process termed cancer immunoediting. To grow and disseminate, the tumor must avoid sterilizing immunity (Bui and Schreiber 2007; Smyth et al. 2006). Tumors developing in the context of an intact immune system may possess immune evasion strategies that are absent from similar tumors developing in an immunodeficient environment. They may lose expression of specific antigens or MHC molecules, enabling them to hide from the adaptive immune system. Mutations in $\beta 2m$, HLA class I, or altered expression of antigen-processing machinery components may diminish or fully eliminate antigen presentation through the MHC class I presentation pathway (Blades et al. 1995; Connor and Stern 1990; Garcia-Lora et al. 2003; Jäger et al. 1997). MHC class II molecules are expressed on some tumor cells and may also be lost, and this has been associated with lymph node metastases in colorectal cancer (Rimsza et al. 2004; Wang 2001; Warabi et al. 2000).

In addition to the well-recognized ability of cytolytic T lymphocytes (CTLs) to lyse tumor cells, Th1 cells have been found in some systems to be effective mediators of antitumor immunity (Pardoll and Topalian 1998; Wang 2001). Th1 cytokines, such as TNF- α , IFN- γ , IL-12, and IL-18, and Th1 cell numbers are increased in colorectal adenomas compared with carcinomas, potentially indicating localized activity of these cells (Cui et al. 2007). However, T cells have mixed roles in tumor development (Muranski and Restifo 2009). Some T-cell cytokines can also promote tumor growth. For example, IL-10 is produced by Th2 and regulatory T cells (Tregs) and its expression correlates with poor prognosis and tumor relapse in some studies (De Vita et al. 1999, 2000; Galizia et al. 2002; Giacomelli et al. 2003; Klein et al. 1999; Yue et al. 1997). IL-10 may act in part by inhibiting tumor-cell apoptosis and promoting vascular growth.

Tumors may contain substantial populations of Foxp3⁺ Tregs or anergic lymphocytes able to suppress effector T-cell responses. Tregs are found in breast, pancreatic, ovarian, head and neck, and non-small-cell lung cancers (Badoual et al. 2006; Curiel et al. 2004; Li et al. 2009; Liyanage et al. 2002). Recently it was shown that Foxp3⁺ Treg cells express VEGFR2, and VEGF blockade could diminish numbers of tumor-infiltrating Tregs (Atanackovic et al. 2008; Li et al. 2006; Suzuki et al. 2010). TGF- β is produced by Tregs as well as other cell types and plays a significant role in immunosuppression (Atanackovic et al. 2008; Gorelik and Flavell 2001; Zou 2005), inhibiting the activation of T cells, NK cells, monocytes, and macrophages (Bierie and Moses 2010; Wrzesinski et al. 2007). TGF- β attenuates the antitumor capacity of tumor-infiltrating CD8⁺ T lymphocytes and can convert potential effector cells into suppressive cell types that also secrete

this cytokine (Selvaraj and Geiger 2007; Shafer-Weaver et al. 2009a). This positive feedback may help shut down effective antitumor immunity. Tumor cells can themselves secrete inhibitory cytokines and chemokines that inhibit effective immunity (Atanackovic et al. 2008). Some express CCR4 ligands, promoting the migration of Tregs as well as Th2 cells to tumor sites, while minimizing the expression of CCR5 ligands that attract Th1 cells (Atanackovic et al. 2008). Some express indoleamine 2,3-dioxygenase (IDO) or other immunosuppressive proteins that generate a tolerogenic environment. IDO inhibits T-cell expansion by tryptophan deprivation (Lob et al. 2009; Zou 2005). The tumor microenvironment is often hypoxic and nutrient depleted, conditions that may further limit effective adaptive immunity.

Pathogens depend on protein structures distinct from their host and therefore possess an array of potential antigenic targets. With the exception of viral-induced tumors, tumor proteins are self-derived. Tissue-specific and differentiation antigens, such as gp100, MART-1, and tyrosine-related protein-1 in melanoma, to which immune tolerance may be incomplete, are therefore prominent targets (Boni et al. 2008; Phan et al. 2003a; Rosenberg et al. 2003). Lymphocytes specific to such antigens are often of low avidity, as higher-avidity cells are deleted (Theobald et al. 1997). Genetic translocations and mutations may create novel antigens within cancers that are absent from the germline. The number of such truly tumor-specific antigens is unclear. Many tumors show considerable genetic instability. However, the genome of others, such as in pediatric acute myeloid leukemia, appear relatively intact (Radtke et al. 2009). Recent efforts aimed at sequencing cancer genomes and assessing for novel splice variants may help resolve the extent to which tumor-specific neoantigens are generated in cancer. Considering the many obstacles to an effective antitumor immune response, it is not surprising that at the time of presentation, the immune system is generally ineffective at controlling tumor growth.

Approaches to Tumor Immunotherapy

Most clinical efforts at tumor eradication have attempted to invoke a host antitumor response. Tumor or tumor antigen vaccination has been tested using a variety of permutations. In contrast to the application of vaccines against infections, in cancer it is necessary to immunize an experienced immune system that may have already been tolerized to tumor. This may in part explain the overall limited efficacy of tumor vaccines to date (Rosenberg et al. 2004).

Other approaches have attempted to resurrect an antitumor immune response through the wholesale

modification of the immune system. In experimental systems, depletion or inactivation of Foxp3⁺ Tregs or TGF- β blockade can promote latent antitumor responses (Kline et al. 2008; Moo-Young et al. 2009; Petrausch et al. 2009; Poehlein et al. 2009). Likewise, both animal models and recent clinical trials have found CTLA-4 blockade to be effective in promoting immune-mediated tumor regression (Peggs et al. 2009; Weber 2007). CTLA-4 blockade with peptide vaccination, however, did lead to autoimmune reactions in melanoma patients (Phan et al. 2003b). Importantly, whereas therapies modifying cytokine and costimulatory pathways can augment responses, they cannot provide for immunologic specificity.

An alternative to manipulating the existing and hampered immune response to tumors is to generate a response capable of targeting tumor cells *ex vivo*. The major advantage here is that the patient's immunologic potential is of little relevance as cells with desirable response properties that are able to target tumor cells are generated outside the host. Transferred NK, NK-T, and $\gamma\delta$ T cells may have an effect in this regard, preferentially recognizing and eliminating transformed cells (Bilgi et al. 2008; Kabelitz et al. 2004; Nakui et al. 2000; Nishimura et al. 2000; Rubnitz et al. 2010; Terabe et al. 2000). However, there is strong theoretical and experimental rationale for administering T lymphocytes as a tumor immunotherapeutic, and a substantial effort is now being made in this direction. The T-cell response is antigen specific, minimizing adverse reactions due to nonspecific immune activation. T cells possess an array of effector functions, including cytolytic activity and cytokine production, which can be manipulated *in vitro* prior to adoptive immunotherapy to allow for the production of T cells optimized for a specific tumor type. T cells can migrate throughout the host, and T cells are not only able to target tumors directly, but to orchestrate other cells, including macrophage and monocytes, to inhibit tumor growth (Yu and Fu 2006).

One approach to acquire tumor-specific T lymphocytes is to expand tumor-infiltrating lymphocytes (TILs) in culture. In initial clinical trials, administered TILs showed objective responses in a significant fraction of patients, but the therapeutic effect was limited and the cells did not persist beyond 2–3 weeks (Chang and Shu 1996; Dudley and Rosenberg 2007; Figlin et al. 1999). In more recent studies, lymphoablative therapy preceding TIL transfer combined with the administration of exogenous IL-2 proved more successful in controlling tumors. The positive effect of prior lymphodepletion suggests that competition for homeostatic niches, cytokines, and activating stimuli otherwise limit the efficacy of adoptive immunotherapy (Gattinoni et al. 2006; Powell et al. 2005). TIL expansion is a lengthy and labor-intensive procedure, and some clinical

trials were abandoned due to insufficient TIL numbers for administration (Figlin et al. 1999).

Importantly, adoptive T-cell immunotherapy has also proven effective in the treatment of virally induced tumors. Autologous Epstein-Barr virus (EBV)-specific lymphocytes can be expanded *in vitro* and have shown success in EBV-positive nasopharyngeal carcinoma, post-transplant lymphoproliferative disease, lymphoma, and Hodgkin's disease (Bollard et al. 2004; Heslop et al. 2010; Liu et al. 2009a; Xu et al. 2006). Although only a small proportion of human cancers have a recognizable viral etiology and possess persistent viral antigens, these results indicate that where high-affinity T lymphocytes specific to genuinely tumor-restricted antigens can be acquired, adoptive immunotherapy can be highly efficacious.

TCR-Modified T Lymphocytes for Tumor Immunotherapy

Acquiring adequate numbers of tumor-specific T lymphocytes for adoptive immunotherapy by selection and outgrowth of host tumor-specific lymphocytes is challenging as these cells may be rare, anergic, or poor-growing and often have low affinity to tumor-specific antigens (Dudley and Rosenberg 2007; Figlin et al. 1999). A more recently adopted approach that circumvents these problems is the direct manipulation of T-cell specificity. This is achieved by transferring TCR cDNA with the desired specificities directly into host T lymphocytes. Most typically, TCR α - and β -chain genes are inserted into retroviral vectors and used to transduce activated host T lymphocytes (Engels and Uckert 2007; Udyavar et al. 2009). Other approaches include lentiviral transduction (Circosta et al. 2009; Zhou et al. 2003), transposon-mediated gene integration (Peng et al. 2009), and direct gene transfer (Cooper et al. 2006).

TCR gene therapy has several advantages in the production of therapeutic quantities of tumor-specific T lymphocytes. Except in the setting of concurrent bone marrow transplantation, T cells for adoptive immunotherapy must be syngeneic. Host T lymphocytes may be readily acquired in large quantity by phlebotomy or apheresis for manipulation. In a short time frame it is therefore possible to accumulate therapeutic quantities of TCR-modified T cells (Heemskerk 2006). The transferred TCRs can be selected to endow T cells with the desired specificities and the T cells can be grown in conditions that optimize their therapeutic potential (Heemskerk 2006; de Witte et al. 2006). TCR gene transfer is subject to some safety concerns. Mismatch pairing of TCR α and β chains between endogenous and gene-transferred TCRs might lead to new specificities and self reactivity (Schmitt et al. 2009).

Leukemia development was observed in X-linked severe combined immunodeficiency recipients of transduced hematopoietic progenitor cells and this led to a reevaluation of the safety of all forms of gene therapy (Pike-Overzet et al. 2007). However, it is noteworthy that a substantial number of studies have employed genetically manipulated mature T lymphocytes, and to date these have had an excellent safety record, without any observed cases of neoplastic transformation (Dossett et al. 2009; Hughes et al. 2005; Morgan et al. 2003).

Numerous studies in animal model systems have attested to the potential of TCR gene transfer in cancer immunotherapy (de Witte et al. 2008; Dossett et al. 2009; Hughes et al. 2005). Limited results have been published in clinical trials and these have been restricted to a single tumor type that is recognized as being particularly immunogenic: melanoma. Results have been encouraging. Either MART-1 or gp100-specific CD8⁺ T cells adoptively transferred into patients in conjunction with IL-2 co-therapy showed efficacy (Johnson et al. 2009; Morgan et al. 2003, 2006). As with prior studies using T cells specific to these melanocyte-specific antigens, autoimmune destruction of normal melanocytes was also seen in some patients. It is important that these trials were merely initial salvos in an effort to treat cancer with TCR-redirectioned T lymphocytes and there is a growing understanding of methods that can be employed to optimize this approach in the future.

Optimizing TCR Expression

Retroviruses are a highly efficient means of gene transfer and transduction efficiencies of greater than 50% may be achieved in human T cells. Published trials using TCR-modified T lymphocytes have used retroviral expression vectors incorporating TCR α - and β -chain genes separated by an internal ribosomal entry site (IRES) (Morgan et al. 2006). The IRES provides a site on mRNA in addition to the 5' mRNA cap to initiate translation. This allows both α and β chain to be encoded by a single cistron. However, whereas the two TCR chains should optimally be produced stoichiometrically, the relative efficiency of translation through the IRES is highly variable. An alternative to using an IRES is the incorporation of a viral 2A sequence between polypeptide segments (Donnelly et al. 2001a, b). These do not form an alternate translation-initiation site. Translation is 5' cap dependent. However, translation pauses at the 2A sequence, and this leads to the release of the nascent polypeptide chain without terminating translation. Although the different 2A sequences have different efficiencies of peptide release, these may be sufficiently high to allow the expression of multiple genes in nearly equivalent quantities from a single cistron. This can

conceivably permit the expression not only of TCR α and β chains, but marker, selection, suicide, or other gene products through a single-transduced construct (Engels and Uckert 2007; Szymczak et al. 2004). 2A sequences leave a short peptide tag at the terminus of protein sequences. Several studies have demonstrated that TCRs bearing 2A sequences are fully functional and, in one comparison, the use of 2A proved superior to an IRES sequence in promoting TCR expression (Alli et al. 2008; Szymczak et al. 2004; Wargo et al. 2009).

$\alpha\beta$ TCRs provide for T-cell specificity through their association with peptide-MHC (pMHC) complexes. T cells express a single rearranged β chain and up to two α chains. With the introduction of an additional α and β chain through TCR gene transfer, up to six $\alpha\beta$ chain pairs can form. The different chains will compete for pairing, although only one pair, the introduced $\alpha\beta$, will be tumor specific. Studies of transfected combinations of α and β chains have demonstrated that association is not stochastic; rather structural features of α and β chains are important for TCR association (Heemskerk et al. 2007). This may be detrimental to TCR gene therapy. Polygamous chain pairing diminishes the density of the tumor-specific TCR on the cell surface. This decreases the avidity of the T cells for tumor antigen and, if the introduced TCR is poorly expressed or pairs poorly, may limit its ability to achieve threshold signaling. As importantly, degenerate TCR association allows the creation of TCRs with new specificities. TCRs are thymically selected for low-affinity self reactivity and the potential for the formation of new combinations of TCRs with high affinity to self is a significant, if hypothetical, concern (Heemskerk 2006; Schmitt et al. 2009). Considering this, selecting TCR α and β chain pairs for immunotherapy predetermined in experimental systems to have high self-association offers clear advantages.

An alternative and potentially superior approach has been to engineer TCRs so that only the desired $\alpha\beta$ pairs can form. Mapping the interface between α and β chains has identified critical residues involved in their association. Several approaches have been used that can enhance TCR pairing. Cysteine substitutions can be introduced in the constant regions of the α and β chains that lead to the formation of a new interchain disulfide bond (Boulter and Jakobsen 2005; Cohen et al. 2006; Kuball et al. 2007). Other approaches include directly linking CD3 ζ to TCR chains (Sebestyen et al. 2008), use of single-chain TCRs (Lake et al. 1999; Zhang et al. 2004), mutating complementary C α - and C β -interacting residues (Voss et al. 2008), and substituting human C α and C β with their murine counterparts (Cohen et al. 2006; Voss et al. 2006). A distinct approach that has been successfully tested is encoding siRNA that specifically downmodulates endogenous TCRs

within siRNA-resistant TCR expression constructs (Okamoto et al. 2009).

The $\alpha\beta$ TCR chains are associated with additional invariant subunits of the TCR, specifically CD3 γ , δ , ϵ , and a homodimeric ζ chain. These incorporate downstream signaling motifs and provide structural stability to the $\alpha\beta$ chains (Feito et al. 2002). Association occurs prior to and is required for the transport of the TCR to the cell surface. Studies of co-expressed TCRs demonstrate competition for CD3 association, and TCR $\alpha\beta$ pairs differ in their ability to compete (Heemskerk et al. 2007). Identifying TCRs that strongly associate with CD3 and are well expressed on the cell surface is therefore an important step in developing new receptors for immunotherapy.

TCR Specificity

Target choice will undoubtedly prove critical to successful TCR gene therapy. Ideally, antigenic targets should be essential for tumor survival so as to minimize the selection of variant proteins (Kammertoens and Blankenstein 2009). They should also be tumor specific, such as those formed due to common mutations, and identifying these must be a priority. However, other antigens that are preferentially expressed on tumors yet are not wholly specific may also be successfully targeted. Examples include MART-1, gp100, and tyrosine-related protein-1, which are expressed in both melanomas and normal melanocytes and have been extensively studied (Rosenberg 1999). Targeting cancers expressing these antigens often leads to the destruction of normal tissue cells as well as tumor cells, and cases of vitiligo or ocular autoimmunity have been well documented (Overwijk et al. 1999; Palmer et al. 2008; Yee et al. 2000; Yeh et al. 2009). Human epidermal growth factor receptor 2 (HER2) drives tumor proliferation and is over-expressed in breast cancer. HER2-directed antibodies (trastuzumab) have given rise to long-lasting tumor clearance and HER-2 is therefore a promising target for TCR gene therapy (Bernhard et al. 2008; Freudenberg et al. 2009). However, HER2 may also be expressed on non-transformed cells, and CTLs recognizing HER2 may also recognize HER3 and HER4. Specificity must be considered in the application of such TCRs (Bernhard et al. 2008; Conrad et al. 2008). Whether TCRs specific to other antigens over-expressed in tumors though present in normal tissues, such as p53 and telomerase, will prove more selective remains to be determined.

Cancer-testis antigens that are normally expressed developmentally but also present in tumor cells may have a diminished potential for cross-reactivity with non-transformed host cells. Over 100 such antigens have been identified (Caballero and Chen 2009). One example,

NY-ESO-1, is expressed in a broad array of tumor types, allowing specific TCRs to potentially serve as an “off-the-shelf” reagent for immunotherapy for patients with different tumor types (Nicholaou et al. 2006). Multiple TCRs have been identified and characterized that recognize an immunodominant NY-ESO-1 epitope (Ebert et al. 2009). Combined use of several TCRs specific to a single epitope in TCR gene therapy may be advantageous if tolerance, survival, and activity of T cells expressing the different TCRs vary. Tumor-specific TCRs need not be derived from host T cells, but can be induced by immunization of humanized mice or alternative species with tumors or tumor antigens (Voss et al. 2006). Humanized TCRs have not been as well studied as humanized antibodies, though partial humanization is achievable and chimeric mouse/human TCRs have been successfully produced (Cohen et al. 2006).

Survival of Adoptively Transferred T Cells

Activated effector T cells, particularly CTLs, are highly dependent upon cytokines such as IL-7, IL-2, and IL-15 for their survival. When cells are expanded in culture, these cytokines are abundant. Their diminished availability upon transfer prompts cellular apoptosis (Brown et al., 2005). In most immunotherapy systems and clinical trials, isolated CTLs are transferred. These cells must compete for endogenous cytokines for survival. Indeed, in clinical assessments of adoptive immunotherapy, the life span of transferred T cells has often been brief (Chang and Shu 1996; Dudley and Rosenberg 2007; Figlin et al. 1999). Immune depletion, such as via radiotherapy, can enhance cell survival, likely by decreasing competition with host T cells for niches in which they may receive critical homeostatic growth signals (Dudley and Rosenberg 2007; Rosenberg et al. 2008). The provision of exogenous cytokines, with IL-2 most often used clinically, also increases the availability of survival signals (Eberlein et al. 1982; Lotze et al. 1986; Mule et al. 1984).

Both lymphodepletion and exogenous cytokine therapy, however, carry their own risks and toxicities. Preferably, T cells should be self-sustaining while remaining active against tumors, even in the setting of a potentially immunosuppressive tumor microenvironment. Modifying the immunologic milieu, for instance with TGF- β or CTLA-4-blockade or depletion of immunosuppressive DCs, may augment adoptive immunotherapy with TCR-modified T cells, and these strategies have shown effectiveness in other immunotherapeutic models (Disis 2009; Nesbeth et al. 2009; Peggs et al. 2009). Another option is providing the transferred cells with the necessary survival and activation signals. T-cell help is essential for optimal CTL formation

and memory cell differentiation. Th cells activate antigen-presenting cells that may secondarily signal into CTLs and directly provide growth factors. They can also induce the local production of chemokines that attract CTLs to sites of antigen expression and thereby increase the effectiveness of CTL responses (Nakanishi et al. 2009; Norris and Rosenberg 2002; Zhang et al. 2009). The tumor microenvironment lacks the inflammatory signals present during infections, where the role of helper T cells in sustaining immune reactivity is well established. Nevertheless, clinical benefit in a mixed Th and CTL response has been observed in various tumor models (de Goer de Herve et al. 2008; Shafer-Weaver et al. 2009b; Wong et al. 2008). Furthermore, in a single report, adoptive immunotherapy with NY-ESO-1-specific CD4⁺ T cells alone showed efficacy in the treatment of melanoma (Hunder et al. 2008). Whether TCR-transduced helper T cells will synergize with similarly TCR-modified CTLs in cancer immunotherapy remains to be determined.

How synergism between Th cells and CTLs can be optimized for different tumor types must also be better defined. Th cells include multiple subtypes, such as Th1 cells that dominantly secrete IFN- γ and promote macrophage activation and Th17 cells that are effective recruiters of neutrophil responses. These cell types can be generated in vitro using specific cytokine and activation regimens (Stockinger and Veldhoen 2007; Zhang et al. 2009). The optimal form of help may differ depending on the pathophysiology of specific tumors, although both Th1 and Th17 cells have proven effective in model systems (Hong et al. 2008; Martin-Orozco et al. 2009; Zhang et al. 2007). Furthermore, much like CTLs, the survival of Th cells depends on adequate stimulation after transfer. One approach is to transduce tumor-specific TCRs into T cells specific to a chronically infecting virus. The virus-specific response may help sustain the T cells that can then secondarily target tumor cells (Pule et al. 2008). Effector T cells derived from central memory T cells (T_{CM}) transfected with tumor-specific TCRs have also been shown to persist for a longer time than their naïve T cell-derived counterparts (Berger et al. 2008). However, further validation of this approach is needed. In the case of CD8⁺ T cells, transferred naïve T cells are therapeutically more active than T_{CM} (Hinrichs et al. 2009).

Manipulating T-Cell Avidity

T-cell response is dependent on the signal received through the TCR. With increasing signaling, cytolysis, cytokine production, and proliferation will sequentially initiate in CTLs. Inadequate signaling can lead to cell death as anti-apoptotic pathways are not adequately induced. However,

too intense a signal may also prompt apoptosis (Brown et al. 2005; Klein et al. 2009). Although multiple aspects of T-cell recognition of pMHC ligand compositely determine avidity, TCR affinity is critical among these. Indeed, TCR mutations that increase affinity may also enhance T-cell reactivity and enable responses to otherwise sub-threshold stimulation conditions (Stone et al. 2009). TCR gene therapy provides the opportunity to engineer TCRs so as to alter their affinity. TCRs engage pMHC through a set of surface-exposed peptide loops called complementarity-determining regions (CDR). Three CDRs are present on each TCR chain: CDR1, 2, and 3. CDR3 is oriented to directly engage antigenic peptide whereas CDRs 1 and 2 are more involved in orienting the TCR on the MHC ligand (Marrack et al. 2008; Varani et al. 2007).

Several approaches have been used to modify TCR affinity. In vitro evolution has been highly successful. Single-chain TCRs are expressed on yeast or through phage display. The parental TCR undergoes random mutagenesis. Affinity-based selection for pMHC is used to identify high-affinity variants (Weber et al. 2005; Zhao et al. 2007). Multiple rounds of mutagenesis and selection have allowed the production of TCRs with affinities up to several thousand-fold greater than the parent receptor. In some cases, the mutations identified in CDR residues have been reintroduced and further refined in TCR $\alpha\beta$ heterodimers that can be used for TCR gene therapy (Richman and Kranz 2007; Robbins et al. 2008). Alternatively, a T-cell display system in which randomly mutated TCR genes are transduced into TCR-deficient T-cell hybridomas has been used to generate high-affinity TCRs (Chervin et al. 2008). High-affinity TCRs can also be generated by site-directed mutagenesis of the CDR1, 2, and/or 3 regions (Chlewicki et al. 2005). We hypothesized that iterative mutagenesis of CDR3 alone could identify critical recognition residues and modify TCR avidity. Using this approach we were able to rapidly create with single amino-acid mutations TCRs with even a >1000-fold increased sensitivity for cognate antigen (Udyavar et al. 2009).

Although the technology for manipulating TCR affinity has matured, the optimal affinity of TCRs for immunotherapy is not yet established. Clearly, TCRs with too weak an affinity will fail to adequately stimulate T lymphocytes. An affinity ideal may be construed from the natural progression of immune responses. Studies of these have indicated that the role of affinity may differ in different T-cell types. CD8⁺ T-cell responses demonstrate progressively enhanced TCR avidity with time after antigen exposure, implying that higher-affinity clones are more fit and able to outcompete those with lower antigen affinities (Kedl et al. 2003; Price et al. 2005). Studies of CD4⁺ T-cell responses have yielded less consistent results, with some but not other studies identifying affinity-based competition

(Fasso et al. 2000; Malherbe et al. 2004). Antigen quantity appears important, with avidity-based competition most prominent when antigen is limited (Blair and Lefrancois 2007; Rees et al. 1999). In this regard, avidity-based selection has been observed with autoreactive diabetogenic T cells, where antigen is derived from an islet cell mass progressively shrinking with disease, but not with encephalitogenic T cells that respond to abundant central nervous system antigens (Amrani et al. 2000; Hofstetter et al. 2005).

One theoretical advantage of high-affinity TCRs is that above an affinity threshold of approximately 1 μ M, TCRs acquire co-receptor independence for response (Holler and Kranz 2003). Therefore very-high-affinity class II MHC-restricted TCRs can stimulate CD4⁺CD8⁺ T cells and high-affinity class I MHC-restricted TCRs can activate CD4⁺CD8⁺ T cells. Such high-affinity TCRs may allow a single-transduced receptor to provide both helper and cytolytic activities, depending on the type of T cell transduced (Udyavar et al. 2009). However, manipulated TCRs may also impose new safety concerns. Studies of in vitro engineered TCRs have indicated that novel specificities may be introduced, particularly new self reactivity (Holler et al. 2003; Udyavar et al. 2009; Zhao et al. 2007). It is therefore essential that altered TCRs are thoroughly validated for the acquisition of new undesirable reactivities prior to clinical application (Donermeyer et al. 2006; Udyavar et al. 2009).

Can TCR Cross-Reactivity be Minimized by Modulating TCR Entropy?

T cells in the human body possess approximately 2×10^7 distinct TCRs that must recognize a much broader array of antigens presented by pathogens and tumors (Arstila et al. 1999). They are able to do this through degenerate recognition of antigen. The CDRs that engage pMHC are not structurally fixed, interacting with pMHC in a “lock-and-key” manner. Rather they possess enormous flexibility and are able to conform to different pMHC structures (Hare et al. 1999; Jones et al. 2008; Wilson et al. 2004). Because of TCR degeneracy, it is estimated that a single receptor can bind as many as 10^6 distinct pMHC complexes.

When a TCR engages pMHC, molecular interactions provide binding energy. This energy may be derived from two sources: changes in the enthalpy or the internal energy of the system and changes in the entropy or the randomness of its constituents. With binding, the TCR structure settles on a conformation best suited for its ligand (Wilson et al. 2004). Consequentially, there is an entropic price when TCRs engage pMHC, and this must be compensated by the enthalpy of binding. TCR mutations can theoretically

increase the total free energy of binding either by increasing the enthalpy of binding or diminishing the loss of entropy with binding. Most structural manipulations increase TCR affinity by mutating the critical CDR residue involved in ligand engagement. An improved fit and bonding between the proteins will enhance the enthalpy of binding. However, because TCRs are selected in the thymus for low-affinity binding to MHC, broadly increasing the enthalpy of binding to MHC without providing additional ligand specificity may convert interactions of too low an affinity to stimulate T cells into stimulatory interactions. This will lead to an altered pattern of TCR reactivity and the acquisition of new specificities, including self specificities.

In contrast to mutations that primarily influence TCR binding enthalpy, it is in theory possible to introduce mutations that enhance affinity or free energy of pMHC binding not by increasing the enthalpy of the reaction, but by decreasing entropy, that is by diminishing TCR flexibility and converting a loose fit for pMHC into a lock and key. These types of mutations would be anticipated to enhance affinity while diminishing the possibility of degenerate recognition. Deciphering the structural features underlying TCR degeneracy and CDR flexibility, and developing methods to enhance affinity by diminishing TCR entropy rather than increasing binding enthalpy, may prove important in the development of high-affinity TCRs for immunotherapy that have improved specificity.

Application of TCR Gene Therapy to Nonmalignant Diseases

The same approaches used to modify immune responses in cancer may also be useful in chronic infections and autoimmune diseases. Similar parameters will be important in the selection of optimal TCR and T-cell types for the adoptive immunotherapy of infectious diseases as cancer, although the presence of easily identifiable pathogen-specific antigens should facilitate therapeutic design (Varela-Rohena et al. 2009).

Immunotherapy of autoimmune diseases with TCR-modified T cells presents distinct challenges. The pathological T lymphocytes in this circumstance are self specific and therapeutic self-antigen-specific T lymphocytes are needed to impart disease selectivity. The most straightforward approach would be to modify regulatory T lymphocytes, directing them against targets of interest. Indeed, adoptively administered Tregs, including one utilizing TCR gene transfer, have demonstrated therapeutic efficacy in many autoimmune model systems and specificity is important for optimal response (Hori et al. 2002;

Huter et al. 2008; Selvaraj and Geiger 2008; Tang et al. 2004; Wright et al. 2009). A variety of regulatory T lymphocyte subsets have been identified, including Tr1, Th3, and Foxp3⁺ Tregs (Verbsky 2007; Workman et al. 2009). Due to the plasticity of T-cell responses, it would be vital to assure that transferred Tregs do not convert into pathogenic forms. Potentially the best-suited cell type for this purpose is natural Foxp3⁺ Tregs (nTregs). nTregs are a distinct T-cell lineage that develops in the thymus in parallel with conventional $\alpha\beta$ T lymphocytes (Workman et al. 2009). Conventional T cells can also adaptively upregulate Foxp3 and acquire regulatory function with stimulation in the presence of TGF- β and IL-2, although these demonstrate less stability when transferred in vivo (Selvaraj and Geiger 2007).

Whether TCR gene therapy with regulatory T cells should utilize Treg-derived rather than conventional TCRs is an important unresolved question. Tregs must receive distinct homeostatic signals compared with conventional T lymphocytes and their predilection for self reactivity may be important in their ability to garner these signals. Indeed, the TCR repertoire utilized by Tregs and conventional T cells is distinct, although it shows some overlap (Hsieh et al. 2006). Importantly, in a study of TCRs used by Tregs in a mouse model for multiple sclerosis, distinct TCR use was observed among regulatory and effector T cells specific to an identical autoantigen (Liu et al. 2009b).

Conclusion

The evidence that adoptive immunotherapy can be used to treat cancer is now incontrovertible, yet the impediments to establishing and maintaining effective antitumor immunity remain substantial. TCR gene therapy shows considerable promise as an adoptive immunotherapy because of its ability to rapidly generate a large population of tumor-specific T cells of defined characteristics. Optimal implementation will require consideration of both T-cell biology and tumor pathophysiology. A concerted approach is needed that will include identifying tumor-specific antigens that are well presented and recognized by T cells, isolating specific TCRs, and, where necessary, modifying these to optimize affinity and response, defining the T-cell types or combinations of T-cell types best suited for the immunotherapy of specific tumors and assessing the suitability of adjunct treatments to ensure those T cells efficiently migrate to the tumor site, can successfully eradicate the malignant cells, and remain viable for a prolonged period. The momentum toward reaching these goals has accelerated in recent years, and it can be hoped that TCR gene therapy will shortly find a place in the armamentarium available to battle cancer.

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