Adoptive immunotherapy: Engineering T cell responses as biologic weapons for tumor mass destruction

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Adoptive T cell immunotherapy is an evolving technology with the potential of providing a means to safely and effectively target tumor cells for destruction.

Targeted destruction of malignancies by enhancing T cell responses is an attractive modality for therapy because it potentially allows for exquisite specificity and potent activity in the elimination of target cells while avoiding toxicities associated with many other standard approaches. However, therapeutic vaccination of cancer patients has been difficult, due in part to the immunocompromised status of patients and immune tolerance mechanisms that result in the deletion or anergy of self-reactive T cells. Adoptive immunotherapy with large numbers of ex vivo expanded effector T cells can overcome many of the regulatory mechanisms that prevent the in vivo generation of effective responses, and provides an opportunity to control the magnitude, function, specificity, and target avidity of the antitumor response. Adoptive therapy can also be used to rapidly determine if potential antigens are valid therapeutic targets and to define the goals that must be achieved for an effective vaccine. Moreover, T cells can be genetically modified prior to administration to instill effector functions and specificities that may not be normally present. This primer discusses the critical steps and strategies being examined for the generation and effective deployment of effector T cells in the treatment of human cancer (Figure 1).

Target selection

There are several criteria that an optimal tumor antigen would possess. Ideally, the target antigen would be expressed in the malignant but not the normal cell type. Also, the target antigen should be required by the tumor cells for growth or survival. This would minimize the risk of evading the transferred T cells, either by mutation or loss of expression of the antigen. Potential targets in this category include epitopes from the BCR/ABL translocation in CML (Groffen et al., 1984), defined p21/ras mutations found in multiple malignancies (Bos, 1988), and oncogenic viral proteins such as HPV proteins in cervical cancer (zur Hausen, 1999) and EBV proteins in Hodgkin's disease and nasopharyngeal carcinoma (Niedobitek et al., 2001). However, most candidate antigens will not fulfill these criteria, but rather will represent nonmutated proteins that are overexpressed or aberrantly expressed by the tumor. Again, proteins important for maintaining the malignant phenotype should preferably be chosen. Quantitatively higher expression of such proteins in malignant cells compared to normal tissues may make it possible to isolate effector cells with T cell receptor (TCR) affinities that recognize and eliminate tumors while ignoring the low levels of antigen expressed by normal tissues, since there should have been little developmental pressure to delete or tolerize such T cells. Overexpressed protein antigens associated with tumorigenicity currently being targeted include the WT1 (Wilms' tumor) gene in leukemias and various solid tumors (Yamagami et al., 1996), and HER-2/neu in breast and ovarian cancer (van de Vijver et al., 1987). Aberrant expression in tumors of proteins found only in normal tissues perceived as immune-privileged provides nearly unique potential target antigens. The prototypes are cancer-testis antigens, such as NY-ESO-1, which have unknown functions but are expressed by transformed cells such as ovarian cancer and melanoma and otherwise are detected only in normal germ cells (Chen et al., 1997). Alternatively, tumor antigens might be selectively targeted if their expression is limited to normal tissues in which the consequences of autoimmune injury would be acceptable, such as with PSA in prostate cancer and normal prostatic tissue (Kuriyama et al., 1982) and MART-1 in melanoma and normal melanocytes (Kawakami et al., 1994). However, the risk of evasion would likely be greater with such antigens, as demonstrated by the selective outgrowth of antigen-loss mutants following immunotherapy of melanoma (Thurner et al., 1999; Yee et al., 2002).

Methods for identifying tumor-specific or tumor-associated antigens that might be targeted continue to improve with technological advances. One approach uses tumor-reactive T cells isolated from the blood or tumor tissue (i.e., tumor-infiltrating lymphocytes or TIL) of cancer patients to screen autologous target cells transfected with genes from a tumor-derived cDNA expression library (Boon et al., 1994; Kawakami et al., 1994). However, this strategy requires the prior isolation of tumor-reactive T cells, and the antigens identified, while a priori immunogenic, may not be ideal targets. Another approach, SEREX, uses antisera from cancer patients to screen tumor-derived expression libraries for antigens that have induced antibody responses, on the presumption that this might be associated with T cell responses (Pfreundschuh, 2000). More than 1500 immunogenic tumor proteins have been identified by this method, but the difficult process of determining which of these antigens can actually elicit $CD8^+$ and/or $CD4^+$ T cell responses remains. Whereas such techniques screen for immunogenic proteins to identify candidate tumor targets, an alternate strategy utilizes gene expression profiles—for example with microarrays or differential displays—to screen tumor transcriptomes to identify candidate genes that are selectively overexpressed in malignant versus normal cell types, many of which can be identified as prooncogenic. The most suitable proteins can then be screened for immunogenicity.

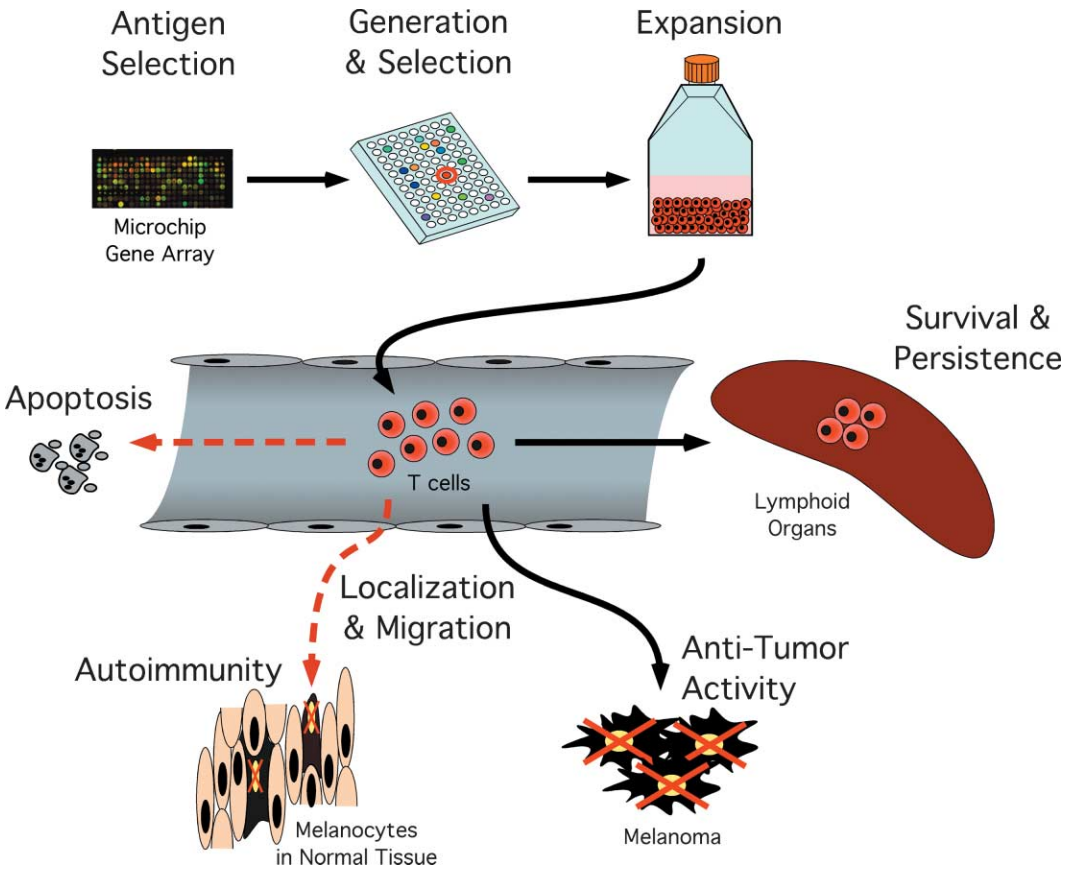

Figure 1. Critical checkpoints and pitfalls in adoptive T cell immunotherapy

Following selection of a target antigen, T cells are stimulated in vitro, and reactive cells isolated and expanded to large numbers. After infusion into patients, the T cells must persist, retain function, and localize to tumor sites to be effective. Failure to support in vivo survival can result in apoptosis of the transferred cells, which interferes with efficacy. Normal tissues may also express the targeted antigen, and strategies to avoid injury to such tissues must be considered.

In vitro generation of effector cells

The next step essential for adoptive immunotherapy is the generation of effector cells. Elucidation of the roles played by cellular and molecular factors in the development of in vivo immune responses has led to improvements in the methods used to produce effector cells in vitro. The generation of CTL effectors ex vivo may uncover responses not evident in the potentially inhibitory in vivo environment. High-avidity CTL reactive with tumor antigens that have escaped thymic deletion might still be rendered nonresponsive (anergized) in the periphery by expression of the antigen in normal adult tissues or in the growing tumor (Lee et al., 1999; Ohlen et al., 2002). Such T cells would not likely respond to in vivo immunization with tumor or peptide-based vaccines, but might respond in vitro in the absence of negative effects from regulatory T cells or inhibitory signals like CTLA-4 (Sutmuller et al., 2001).

The generation of T cells for tumor therapy has generally involved the isolation and expansion of tumor-reactive CTL from patients. A method used in several clinical trials utilizes T cell lines derived from the in vitro culture of cells infiltrating tumor tissue in extremely high concentrations of IL-2 without stimulation with a selected target antigen (Dudley et al., 2002b). While a significant amount of clinical data have been generated with such TILs, polyclonal cells generated under such nonphysiologic conditions may have functional deficiencies limiting efficacy, and the inability to adequately characterize the effector population impedes rational improvement of this methodology.

Generation of effector cells specific for particular target antigens requires in vitro stimulation of peripheral T cells with both antigen presenting cells (APC) and a source of antigen (e.g., actual tumor cells, tumor lysate or protein, or a synthetic peptide, mRNA, or DNA within a shuttle vector). Isolating CTL reactive to a tumor-associated target antigen can be difficult if the antigen is expressed during normal development, since most or all of the high-avidity antigen-reactive $CD8^+$ T cells may have been deleted from the repertoire. To maximize the chances of successfully activating and expanding rare reactive cells, APCs expressing "optimal" costimulatory signals (e.g., mature dendritic cells or artificial APC) are now commonly being used. In addition, supplemental cytokines or additional signals normally derived from APC or $CD4^+$ T helper cells that may affect the quality of effector cell generation are being studied (e.g., IL-7 for naïve T cell survival and IL-15 for expansion of primed $CD8^+$ T cells) (Janssen et al., 2003; Lu et al., 2002; Lynch and Miller, 1994).

Ex vivo culture techniques can be used to generate antigen-

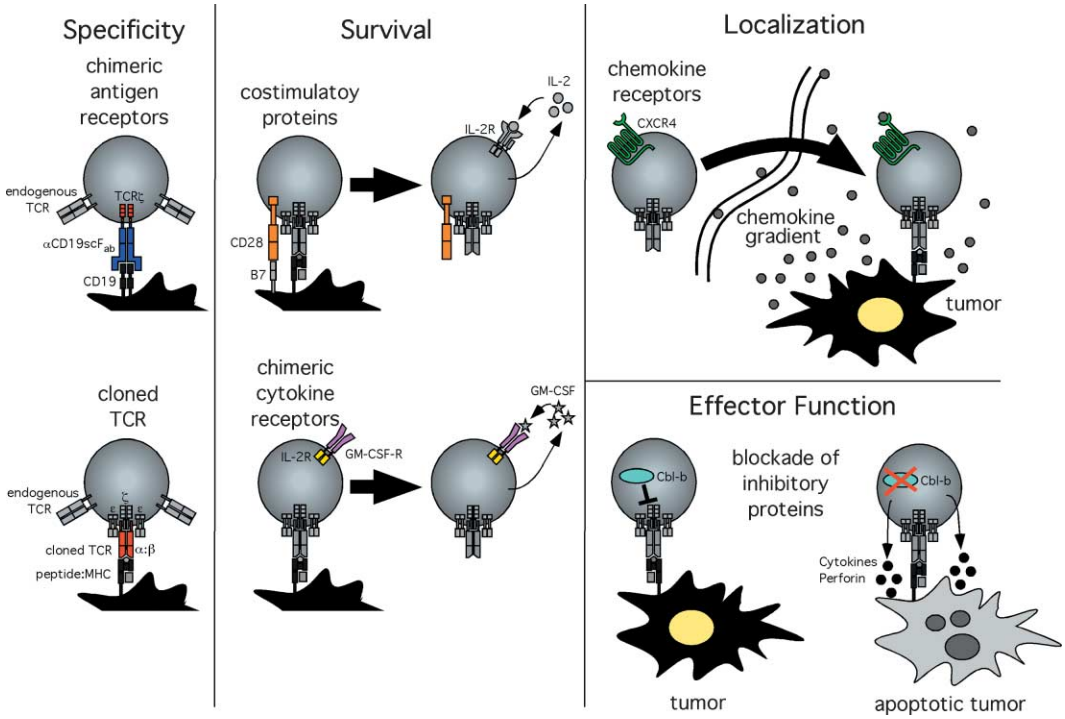

Figure 2. Potential genetic modifications of T cells to enhance adoptive immunotherapy

Specificity: T cells can acquire the desired specificity and affinity for the selected target antigen by introduction of either defined TCR chains or chimeric antigen receptors with antigen binding domains fused to intracellular signaling domains.
Survival: Regulated growth/survival signals delivered following target recognition can be provided by restoring costimulatory signals via CD28 or introducing chimeric cytokine receptors that can be triggered by cytokines produced by T cell activation.
Localization: For tumor eradication, T cells can be directed to tumors in tissues by expression of tissue-specific chemokine receptors such as CXCR4.
Effector function: Intracellular signals such as those mediated via Cbl-b that normally inhibit T cell activation signals may be blocked to sustain effector functions, including cytokine production and cytolysis, promoting effective antitumor responses.

specific effector cells either as components of enriched T cell lines or as homogeneous populations of T cell clones. T cell lines are easier to generate and can potentially include both $CD4^+$ and $CD8^+$ antigen-reactive effector T cells, and the administered population of tumor-reactive cells may retain greater in vivo proliferative potential due to having undergone fewer divisions ex vivo. However, there can be unpredictable variability among the T cell lines generated, and the heterogeneous effector populations can only be imprecisely characterized. By contrast, the use of homogeneous populations of CTL clones allows for the administration of effector cells with well-defined specificity, avidity, effector function, and magnitude, which facilitates analysis of the basis for therapeutic success or failure. Clones are commonly generated by growing colonies derived from individual CTL present in T cell lines plated in limiting dilution. Recently developed techniques for identifying and isolating rare specifically reactive T cells from larger populations—such as the use of peptide-MHC tetramers or a bispecific antibody to capture T cells producing IFNγ in response to specific stimulation—are greatly improving the efficiency of generating both T cell lines and clones (Becker et al., 2001; Keenan et al., 2001).

Ex vivo effector cell expansion

Once CTL lines or clones have been generated and characterized, the cells must be expanded to numbers sufficient for clinical use. Methods continue to be developed and improved to provide optimal cycles of stimulation and growth in vitro by providing signals via TCR engagement, accessory molecules, and growth factors. A standard protocol for expanding CTL clones used in several clinical trials employs TCR stimulation to activate T cells plus allogeneic feeder cells for costimulatory support and 50 IU/ml IL-2 as a growth factor, typically yielding up to 1000-fold expansion of T cells during a two-week cycle (Riddell and Greenberg, 1990). Alternative methods also involve providing some form of TCR stimulation and costimulatory signals via activating antibodies presented on plastic beads or artificial APCs (AAPCs) in the presence of IL-2 (Garlie et al., 1999). In fact, recent studies with such AAPCs have suggested that providing antiapoptotic signals via ligation of 4-1BB on $CD8^+$ cells in addition to costimulatory and TCR signals may enhance cell yield (Maus et al., 2002). Alternatively, TILs have been expanded by culturing in >100× higher levels of IL-2 (Dudley et al., 2002b), which yields large cell numbers and can bypass the requirement for TCR stimulation but may result in abnormal cell function and survival.

IL-2, the canonical T cell growth factor, promotes the cell cycle progression of previously activated and cycling T cells; however, it also predisposes the cycling cells to activation-induced cell death (AICD) if the TCR is engaged, presumably as a form of negative feedback control of expanding T cells (Zheng et al., 1998). IL-15, which signals through the same β and γ receptor chains as IL-2, may rescue cells from AICD. Recent

studies by our group (unpublished data) and others have suggested that IL-15 may better promote expansion and survival of CTL (Brentjens et al., 2003; Lu et al., 2002). This improved survival with IL-15 may be due to intracellular signaling properties of the unique α chain of the IL-15 receptor (IL-15Rα) not provided by the nonsignaling IL-2Rα chain (Bulfone-Pau et al., 1999), or to IL-2 and IL-15 delivering qualitatively or quantitatively different signals through the shared signal-transducing β and γ chains as a consequence of the kinetics or duration of the signals. For example, signaling by IL-15 but not IL-2 may upregulate the antiapoptotic protein Bcl-x_L (Brentjens et al., 2003).

Enhancement of in vivo survival of effector T cells

Transferred T cells must persist in vivo to mediate therapeutic efficacy. Clinical trials with $CD8^+$ CTL clones have demonstrated persistence of infused T cells for prolonged periods if in vivo antigen burden is low, but rapid disappearance of CTL if significant levels of antigen are present (Walter et al., 1995; Yee et al., 2002). Both cotransfer of $CD4^+$ T cell help and administration of IL-2 in vivo have been shown to prolong the survival of CTL (Blattman et al., 2003; Yee et al., 2002). Similar to strategies being pursued to optimize in vitro T cell expansion and survival, providing additional signals in vivo via costimulatory molecules such as 4-1BB or CD28 or via cytokines such as IL-7, IL-15, and IL-21 may prove useful for prolonging the in vivo survival and therapeutic efficacy of transferred T cells (Lu et al., 2002; Lynch and Miller, 1994). However, in vivo regimens will need to be defined that are both effective and avoid significant toxicity.

An alternative strategy to enhance CTL survival in vivo may be to transfer T cells into lymphopenic hosts, which takes advantage of the natural host homeostatic response to promote expansion of the remaining T cells. Prior lymphodepletion has improved the persistence and therapeutic efficacy of transferred $CD8^+$ cells in murine models of adoptive therapy (Dummer et al., 2002) and has appeared promising during treatment of patients with advanced metastatic melanoma (Dudley et al., 2002a). However, the magnitude and duration of lymphodepletion and short- and long-term safety profiles of this approach still require evaluation.

Building a better T cell

While modifying costimulatory signals or cytokines provided either in vitro or in vivo may result in greater numbers of effector cells that survive longer after infusion, it may also be desirable to alter specific characteristics of the cells generated in order to improve their efficacy. Advances in genetic engineering, in concert with a greater understanding of the immune response at a molecular level, have fostered efforts to genetically modify effector cells to provide desired antigen specificity, and enhanced effector cell survival, localization, function, and safety (Figure 2).

Specificity

One obstacle to broadly pursuing T cell transfer as a therapeutic modality is the requirement to generate sufficiently high-avidity tumor-reactive CTL de novo for every patient. This problem could be solved if an autologous T cell found to be effective and safe in one patient could be stored and used in other patients with tumors expressing the same antigen. Although major and minor histocompatibility disparities between individuals make this impossible, molecular technologies have now made it possible to clone and then express the TCR chains as a means to engineer autologous effector T cells with the desired specificity/avidity. Thus, a library of effective TCR α and β chain pairs could be cloned into retroviral vectors, and the appropriate TCR expressed in T cells of patients with tumors expressing the same antigen and HLA-restricting allele (Clay et al., 1999; Cooper et al., 2000; Stanislawski et al., 2001).

This approach can also be adapted to produce TCRs with high affinity for targets even if T cells with such receptors have been deleted or tolerized. T cells with low affinity can readily be generated, and the TCR chains can then be cloned, mutagenized in vitro, displayed on the surface of yeast (Holler et al., 2000) or on retrovirally transduced T cells (Kessels et al., 2000), and selected for the desired increase in affinity via a high-throughput screening technology such as binding to peptide-MHC tetramers. Another strategy for identifying high-affinity TCRs is screening for T cells that can recognize allogeneic peptide/MHC complexes, since such T cells have not been subjected to negative selection during development. For example, $HLA\text{-}A2^+$ stimulator cells pulsed with peptides can be used to generate alloreactive $CD8^+$ clones from HLA-A2-negative individuals that are then screened to isolate the CTLs that recognize the A2 alloantigen only if it contains the specific peptide, and these CTLs used as TCR donors (Stanislawski et al., 2001). Alternatively, xenogeneic TCRs can be isolated by stimulating mice transgenic for the human HLA-A2 molecule with human tumor proteins (Stanislawski et al., 2001). An additional method of providing effector cells with high-affinity receptors requires generating monoclonal antibodies to target cells and screening for specificity for peptide/MHC complexes. This approach takes advantage of the fact that antibodies have higher affinities than TCRs, and that the antibody repertoire has not been tolerized against TCR epitopes. Such "MHC-restricted" antibodies can be engineered to behave as TCRs by constructing chimeric antigen receptors (CARs) composed of the antigen binding domain of the antibody as the extracellular portion fused with an intracellular activation domain, thus linking antigen recognition with T cell activation (Chames et al., 2002). However, high-affinity receptors generated by any of these approaches will have to be carefully evaluated for the potential to induce autoimmunity by recognition of the target antigen at low levels on normal tissues or by crossreactivity with other self-antigens (Holler et al., 2003).

Tumors may evade even effector cells with high-affinity receptors for obligate oncoproteins by disrupting the antigen processing or presentation machinery. Thus, CARs that recognize intact membrane proteins are also being designed to recognize tumor antigens independent of antigen processing or MHC expression. Such CARs consist of an extracellular domain (e.g., either a ligand for a membrane molecule or a single chain antibody) that can bind a membrane antigen on the tumor cell, fused to a cytoplasmic signaling domain such as the TCR ζ chain (Eshhar et al., 1993; Roberts et al., 1994). A large number of different "tumor-associated" antigens including CEA, PSMA, and CD19 have already been successfully targeted experimentally using CARs (Brentjens et al., 2003; Sadelain et al., 2003). One potential drawback of this approach is that since CARs neither promote assembly of the complete TCR signaling complex nor mimic the classical on/off kinetics of TCR-peptide-MHC interactions (Kalergis et al., 2001), the atypical strength and duration of activation signals could result in T cell anergy/exhaustion or apoptosis. At this point there are no clear rules of engagement regarding the ideal CAR affinity or costimulatory requirements for in vivo efficacy, and this may need to

be determined for each CAR generated.

In vivo survival

T cells can also be modified to express autocrine growth factors including IL-2 or IL-15 to sustain survival following transfer. However, cytokine expression not coordinately regulated with antigen stimulation could result in T cell dysfunction or apoptosis as well as cytokine-mediated toxicity. Signaling through the costimulatory molecule CD28 concurrent with T cell recognition of antigen would synchronize endogenous production of IL-2 with TCR signals. Although differentiation of $CD8^+$ cells to effector cells is generally associated with loss of CD28 expression (Appay et al., 2002), recent studies from our group have shown that reexpression of CD28 in human effector CD8 T cells, via a retroviral vector, results in the restoration of regulated IL-2 production (unpublished data). Unfortunately, tumors rarely express CD80 or CD86, the ligands for CD28, rendering this approach ineffective in a majority of cases. One solution to this problem would be to attach intracellular signaling domains from CD28 to the signaling TCR ζ domains of CARs, which can then provide both TCR and costimulation signals (Sadelain et al., 2003). Alternatively, chimeric cytokine receptors (CCRs) may be used to synchronize antigen stimulation and cytokine signals. One such CCR employs the extracellular domain of the GM-CSF receptor fused to the intracellular signaling domains of the IL-2 receptor and utilizes the normal production of GM-CSF following antigen recognition by effector $CD8^+$ T cells as a regulated autocrine growth factor. This chimeric receptor has been shown to deliver IL-2 prosurvival signals to T cells and enhance T cell proliferation in tumor-bearing mice (Cheng et al., 2002). Thus, delivery of prosurvival proliferation signals to T cells linked to target recognition in vivo may provide a means to improve therapeutic efficacy.

Localization

Effective immunotherapy also requires localization of transferred CTL to tumor sites. Direct injection of CTL into tumors has met with limited success and would only be applicable to accessible, localized tumor masses. For other tumor sites, preferential migration/homing of transferred T cells by enhancing the expression of adhesion molecules or chemokine receptors may be a more viable strategy. For example, introduction of the chemokine receptor CXCR4 into T cells may be useful to target CTL to bone marrow for the treatment of leukemias or metastatic tumors growing in the milieu of marrow stromal cells which produce SDF-1, the ligand for CXCR4 (Peled et al., 1999). Similarly, introduction of CXCR5 or CXCR2 to T cells might be used for targeting CTL to follicular lymphoma cells which produce CXCL13 or melanoma cells which produce CXCL1, respectively (Husson et al., 2002; Kershaw et al., 2002). As our insights into the mechanisms of tissue-specific homing increase, directing the migration of effector T cells to desired sites should become an increasingly important aspect of therapy design.

Effector function

During prolonged antigen stimulation with large tumor burdens, $CD8^+$ T cells may become functionally impaired (manifested by decreased cytokine production and lytic activity) and subsequently deleted. Thus, methods to enhance the in vivo function of transferred CTL may be required for effective therapy. The surface receptor NKG2D has been shown to deliver potent activation and costimulatory signals to T cells (Bauer et al., 1999), and overexpression of NKG2D in CTL might provide a means to augment TCR signal strength following target recognition. Importantly, the ligands for NKG2D, MICA and MICB, are stress-induced and often expressed on tumor cells (Groh et al., 1999). Alternatively, targeted decreases in negative intracellular signals, by retroviral transfer of small inhibitory RNA (siRNA) to decrease endogenous mRNA levels or expression of dominant-negative versions of suppressive proteins, may be useful to enhance CTL function. The list of candidate inhibitory protein targets is long, including cell-surface proteins (CTLA-4, PD-1), cell cycle regulators (p27kip), kinase phosphatases (SHP-1), and adaptor proteins (SOCS- & Cbl-family members). Our lab has pursued decreasing levels of Cbl-b, an E3-ubiquitin ligase, in T cells. Consistent with studies in Cbl-$b^{-/-}$ mice (Chiang et al., 2000), downregulation of Cbl-b in immortalized T cell lines and in human T cells results in a decreased threshold for TCR signaling and in the ability to produce IL-2 and proliferate in response to antigen alone, effectively bypassing the requirements for CD28 costimulation during T cell activation (unpublished data). Depending on the requirements for effective therapy in specific settings, it may be possible to "tailor" levels of selected intracellular proteins and thus direct different effector functions for maximum therapeutic efficacy.

Safety

Various techniques are being developed to allow the selective elimination of infused cells in the event of undesirable toxicity. The primary potential toxicity associated with adoptive T cell immunotherapy is injury of normal tissues also expressing the targeted antigen. Autoimmunity may be expected but tolerable, as when melanoma-specific CTL cause vitiligo (Yee et al., 2000), or potentially dangerous, as might occur if the administration of T cells expressing CD19-specific CARs led to B cell aplasia (Brentjens et al., 2003). Alternatively, autoimmunity against other (nontargeted) self-antigens could result from the use of T cell lines rather than clones in therapy, particularly if in vitro strategies are used that overcome tolerance and expand rare endogenous autoreactive CTL. Finally, there is also a potential risk of autoimmunity with T cells expressing introduced TCR chains, as alternative pairing of endogenous and transduced TCR α and β chains could yield an autoreactive receptor.

An additional risk associated with the genetic modification of effector cells is the possibility of oncogenesis. As demonstrated recently (Marshall, 2003), the introduction of recombinant genes into cells can lead to leukemias in human patients due to the unintentional activation of oncogenes. Using homogeneous populations of transduced CTL clones rather than lines has the advantage of allowing for efficient screening for a potentially oncogenic integration event prior to infusion. However, the risk of toxicity or safety may not always be easy to define, and safety features may need to be incorporated into the engineering of effector cells.

Introducing a conditional "suicide gene" into effector T cells is the most common method used to confer the ability to selectively eliminate the transferred cells should toxicities occur. Initially, genes derived from pathogens such as the HSV thymidine kinase (TK) gene were utilized, since cells expressing this protein convert the antiviral drug ganciclovir into a lethal product (Springer and Niculescu-Duvaz, 2000). Limitations with this strategy became apparent in clinical trials, however, as it was discovered that not only were slowly dividing transferred T cells expressing TK not efficiently eliminated, but that host $CD8^+$ T cell responses to the viral protein led to selective and often rapid elimination of the transferred T cells (Riddell et al., 1996; Verzeletti et al., 1998). Therefore, recent endeavors have

focused on inducible suicide genes derived from the endogenous apoptosis pathway. One such approach consists of a chimeric molecule engineered to induce cell death via the Fas pathway after the binding of an otherwise nontoxic drug that causes multimerization (Thomis et al., 2001). As the chimeric molecule is derived from human proteins instead of a pathogen, chances of inducing an immune response are minimal. In addition, induction of cell death is not dependent upon active cell cycling. Alternative "safety switches" include other inducible death signals such as multimerizable caspases or a tetracycline-inducible toxin.

Concluding remarks

T cell therapy has already shown promise in treating several diseases, including CMV, EBV, melanoma, and leukemia (Dudley et al., 2002a; Rooney et al., 1998; Walter et al., 1995; Warren et al., 1998; Yee et al., 2002). In vitro technologies are making it increasingly possible to identify tumor antigens and to isolate and expand T cells reactive with such proteins. In addition, the availability of recombinant cytokines and reagents to trigger costimulatory molecules are providing means to promote the in vivo survival and function of transferred cells. Finally, advances in molecular immunology and genetic engineering are allowing the introduction of an increasing number of novel genes into T cells that can alter and/or enhance effector cell survival, localization, function, and safety. With the current pace of progress, reliable methods for engineering an effective immune response against cancer should soon become a clinical reality.

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