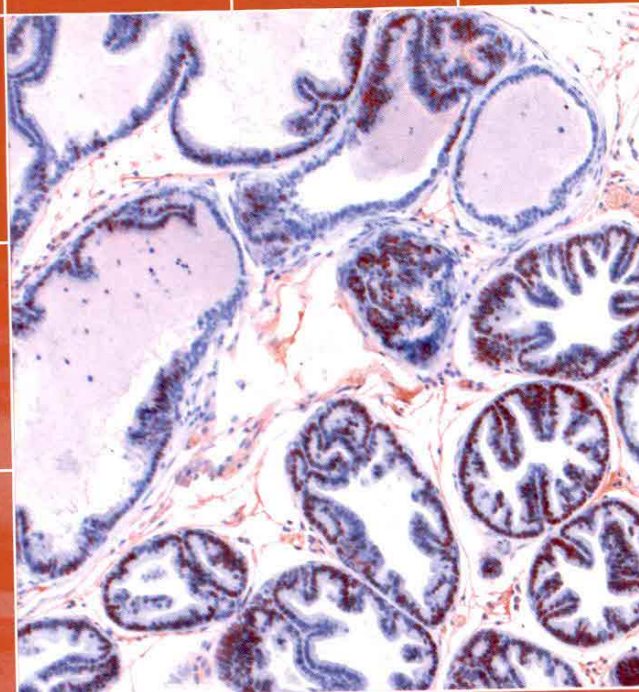


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April 2009

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Premalignant lesions in a mouse model for adoptive T cell therapy of prostate carcinoma. The figure shows the development of early lesions in prostate sections of TRAMP mice as revealed by staining with an antibody specific for the SV40 large T antigen. Administration of engineered T cells specific for the SV40 antigen has been shown to highly reduce disease progression in this preclinical model for adoptive T cell therapy. (Courtesy of Ton Schumacher, NKI, Amsterdam.)



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Adoptive transfer of virus-specific and tumor-specific T cell immunity

Carolina Berger¹, Cameron J Turtle¹, Michael C Jensen² and Stanley R Riddell¹

The adoptive transfer of T cells isolated or engineered to have specificity for diseased cells represents an ideal approach for the targeted therapy of human viral and malignant diseases. The therapeutic potential of adoptive T cell therapy for infections and cancer was demonstrated in rodent models long ago, but the task of translating this approach into an effective clinical therapy has not been easy. Carefully designed clinical trials have evaluated the transfer of antigen-specific T cells in humans, and provided insight into the barriers to efficacy and strategies to improve T cell therapy. The importance of altering the host environment to facilitate persistence and function of transferred T cells and intrinsic properties of T cells that are selected or engineered for therapy in determining their fate *in vivo* are key issues that have recently emerged and are informing the design of the next generation of clinical trials.

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Introduction

An intact and functional T cell compartment is critical for effective immunity to pathogens and there is evidence that T cells can participate in the control and elimination of tumors [1,2]. Studies in rodent models of cancer and infectious diseases have demonstrated that the adoptive transfer of T cells of defined antigen specificity can establish or augment immunity and eradicate malignant or infected cells. Adoptive T cell transfer also has therapeutic activity against human viral infections in allogeneic hematopoietic stem cell transplant recipients, a setting in which virus-specific T cells can be readily isolated and expanded from the immunocompetent donor [3–5]. It was initially perceived that the major

obstacle for applying T cell therapy to human malignancies would be the requirement to isolate and expand tumor-reactive T cells to sufficient numbers to modulate T cell immunity. The identification of tumor associated antigens (TAA) and refined culture techniques have overcome this obstacle for selected tumors such as melanoma. However, in initial studies the infusion of large numbers of T cells or T cell clones specific for TAA failed to completely eradicate tumors in the majority of patients, at least in part due to the short persistence of the transferred T cells *in vivo* [6–9]. This review will summarize insights that have been derived from clinical trials of T cell immunotherapy that have led to progress in developing improved regimens for establishing a durable and functional T cell response using adoptive T cell transfer.

T cell therapy for opportunistic virus infections and virus induced malignancy

A notable success of adoptive T cell transfer is its use to prevent or treat opportunistic virus infections in allogeneic hematopoietic stem cell transplant (HCT) recipients. Regimens for HCT often employ myeloablative doses of chemotherapy and radiation to treat the underlying malignancy and facilitate engraftment of donor stem cells, and either administer immunosuppressive drugs post transplant or deplete T cells from the donor stem cell graft to prevent graft-versus-host disease (GVHD) [10]. These treatments result in a prolonged functional and/or numerical deficit of T cells, and render HCT recipients susceptible to life-threatening infection, both from endogenous latent viruses that reactivate after HCT and from acute community acquired viruses.

Cytomegalovirus

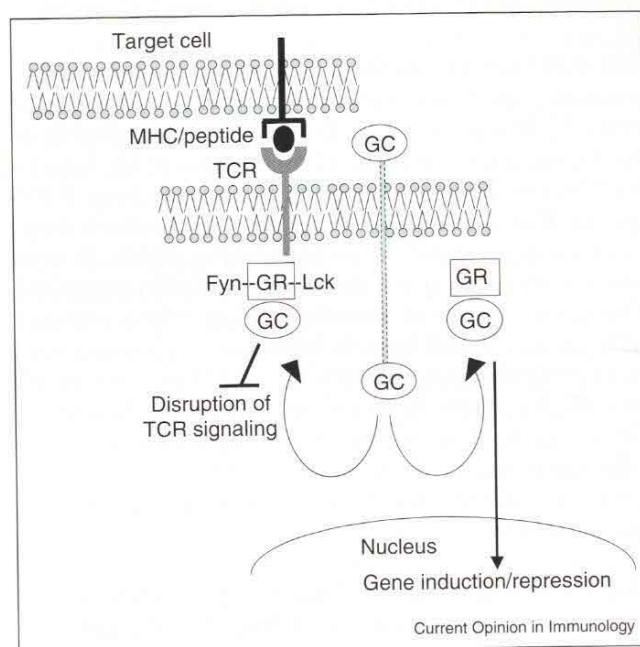
Reactivation of latent cytomegalovirus (CMV) in allogeneic HCT recipients remains a significant cause of morbidity and mortality despite antiviral drug therapy [11]. The finding that progressive infection with CMV correlated with deficient CMV-specific CD8⁺ and CD4⁺ T cell responses suggested that the adoptive transfer of CMV-specific T cells isolated from the immunocompetent donor might be used to restore protective immunity in the recipient. The initial studies of adoptive immunotherapy for CMV infused CD8⁺ T cell clones or polyclonal T cell lines that were derived from the donor and selected for recognition of CMV-infected cells and lack of cross reactivity with recipient alloantigens [5,12,13]. The enrichment and cloning of CMV-specific T cells required

prolonged culture but avoided a risk of GVHD, which is observed frequently if unselected donor T cells are administered to HCT recipients. These studies demonstrated that CD8⁺ and CD4⁺ CMV-specific T cells could be adoptively transferred to patients early after HCT with minimal toxicity, and that the transferred cells could persist and function *in vivo*, and control infection [5,12,13].

Despite this encouraging data, the complex culture methods used to isolate CMV-specific T cells limited the broad and timely application of this approach. More recent efforts have been directed at designing methods to select CMV-specific T cells directly from donor blood. Immunomagnetic selection of antigen-specific T cells has been developed based on binding of tetrameric HLA class I molecules folded with CMV peptides, or the capture of T cells that secrete interferon gamma after antigen stimulation [14,15]. The infusion of remarkably small numbers of donor-derived CD8⁺ T cells selected for binding to HLA class I tetramers containing CMV pp65 or IE-1 peptides to allogeneic HCT recipients with CMV reactivation restored T cell responses to these CMV antigens and reduced the need for antiviral drug therapy [3].

Adoptive T cell therapy for CMV is not uniformly successful in protecting HCT recipients from progressive CMV infection. It is increasingly clear that adoptive transfer of CMV-specific T cells is ineffective in patients that require high doses of glucocorticoids to treat GVHD, which occurs in at least 35% of HLA identical allogeneic HCT recipients that receive a non T cell depleted stem cell graft [16]. Glucocorticoids impair T cell antigen receptor signaling and cytokine production, and promote T cell apoptosis, and these effects are mediated through binding to the glucocorticoid receptor (GR), activation of GR-responsive immunosuppressive genes and inhibition of proinflammatory genes [17,18] (Figure 1). Nongenomic effects of the GR in T cells have also been described, including a physical interaction of unligated GR with the TCR complex, which is disrupted by glucocorticoid binding resulting in impaired activation of Lck and Fyn [19] (Figure 1). Thus, patients that receive prolonged treatment with glucocorticoids have frequent reactivations of CMV and are at high risk of toxicity from antiviral drugs and for the development of drug resistant viral variants. In a clinical trial at the Fred Hutchinson Cancer Research Center, the infusion of CD8⁺ and CD4⁺ CMV-specific T cell clones failed to restore persistent functional T cell immunity in patients that received prednisone, and these patients remained at risk for CMV viremia. These results suggest that glucocorticoids will be useful for reversing toxicity that might develop after adoptive T cell therapy but pose a problem for reconstituting virus-specific immunity in HCT recipients. T cells can be engineered to be resistant to glucocorticoids by interfering with GR expression by introduction of siRNA or by gene editing

Figure 1



Glucocorticoids exert negative effects on adoptively transferred T cells through both nongenomic and genomic mechanisms. The non-ligated glucocorticoid receptor (GR) is associated with the T cell receptor signaling complexes containing Fyn and Lck. Immediately upon binding of the GR to glucocorticoid (GC), these multiprotein complexes are disrupted leading to failure of downstream signaling after engagement of the TCR by antigen. Binding of GC to the GR in the cytoplasm also leads to translocation of the bound receptor to the nucleus and regulation of a number of glucocorticoid responsive genes, including repression of proinflammatory genes such as IL-2, and activation of immunosuppressive genes such as MAPK phosphatase-1, and interleukin 10.

with zinc finger nucleases [20]. The latter approach is now being evaluated in animal models, and may enable the restoration of a protective glucocorticoid resistant CMV-specific T cell response in this high-risk group of patients.

Epstein Barr virus

Severely immunocompromised solid organ and HCT recipients, particularly those that receive a T cell depleted HCT or T cell depleting antibodies to treat GVHD or organ graft rejection, may develop an Epstein Barr virus driven lymphoproliferative disease (EBV-LPD), consisting of EBV-infected B cells that express the highly immunogenic EBNA-3A, 3B, and 3C proteins. Rooney and colleagues have isolated polyclonal EBV-specific T cells containing variable numbers of CD8⁺ and CD4⁺ T cells from the blood of HCT donors by repeated *in vitro* stimulation with EBV transformed B cell lines that express the EBNA proteins, and administered these T cells to the respective recipients with EBV-LPD or at high risk for EBV-LPD. Adoptive T cell therapy targeting EBV was highly effective, both for promoting tumor regression in

patients with established EBV-LPD and preventing the development of LPD when used as prophylaxis [4,21]

EBV-LPD can be rapidly progressive and similar to the situation with CMV, the time required to isolate and expand EBV-reactive T cells is a significant obstacle to the routine use of adoptive T cell therapy. Several groups are developing cryopreserved banks of polyclonal EBV-specific T cells from HLA typed volunteer donors so that a cell product could be immediately available to treat severe EBV infections in unrelated transplant recipients. The initial results of infusing partially HLA matched EBV-specific T cell lines to immunocompromised solid organ allograft recipients with EBV-LPD are surprisingly encouraging, both in terms of safety and therapeutic efficacy [22*]. The degree of immunodeficiency in the recipient is apparently sufficiently severe that the allogeneic T cells are not rejected before mediating anti-tumor activity.

EBV is also associated with a number of malignancies that occur in immunocompetent individuals. A subset of Hodgkin's disease contains EBV genomes and express a limited number of weakly immunogenic EBV proteins, including LMP-2. Bollard *et al.* have used dendritic cells engineered to express LMP-2 as antigen presenting cells to expand autologous LMP-2-specific T cells from patients with Hodgkin's disease [23]. The adoptive transfer of polyclonal T cells containing both CD4⁺ and CD8⁺ LMP-2 specific T cells augmented LMP-2 specific T cell immunity and promoted tumor regression in a subset of these patients [23]. Studies are also in progress to develop adoptive T cell therapy for the subset of nasopharyngeal carcinomas that are EBV positive [24].

Other opportunistic viruses

Effective drug therapy is not available for several other viruses that cause morbidity after allogeneic HCT and might be amenable to adoptive T cell therapy, including adenovirus, community respiratory viruses, and BK virus [25,26]. Our knowledge concerning the antigen specificity and protective capacity of T cell responses to these viruses in humans is incomplete, and the frequency of T cells in donor blood is typically much lower than for latent viruses such as CMV and EBV. Nevertheless, efforts are being made to derive T cell products that contain an expanded repertoire of virus specificities and could be used in adoptive therapy. Leen *et al.* have developed a culture method in which a recombinant adenovirus that encodes the CMVpp65 protein is used to infect EBV-LCL for use in stimulating T cells from HCT donors [27*]. This results in the simultaneous expansion of T cells specific for adenovirus, CMV and EBV, and the infusion of such T cells into HCT recipients augmented responses to all three viruses and promoted virus clearance. It is likely that additional viral antigens could be incorporated into such culture systems to hasten immune

reconstitution to the most prevalent viral pathogens after HCT.

T cell therapy for non-opportunistic persistent viruses

The efficacy of adoptive T cell therapy for viral infections in immunocompromised hosts raises the prospect of using T cell therapy to boost partially effective responses to human immunodeficiency, hepatitis C, and hepatitis B viruses that cause a chronic persistent infection. Early efforts to boost HIV-specific CD8⁺ T cell responses by adoptive transfer were unsuccessful due to short-term persistence of the transferred T cells in individuals with replicating virus [28]. We now know that CD8⁺ and CD4⁺ virus-specific T cells in these chronic infections are characterized by a progressive loss of function and viability related to upregulation of inhibitory molecules such as PD-1 and Tim-3 [29–31]. Thus, the infusion of autologous T cells engineered to recognize these viruses by introduction of virus-specific TCR genes [32,33], combined with inhibitors of PD-1 or Tim-3 signaling pathways might improve the quantity and function of antiviral T cells. This strategy may carry a risk of immunopathology, particularly if the antigen load is excessive at the time T cell therapy is administered. An approach that is being developed to restore the CD4 deficiency in HIV infection is to engineer autologous CD4 T cells for adoptive transfer that lack the CCR-5 co-receptor for HIV entry using zinc finger nucleases to permanently disrupt the CCR-5 coding sequence [34**].

Tumor-specific T cell therapy

Adoptive T cell therapy for human malignancy has proven to be more challenging and less effective than for opportunistic viral infections. This reflects several obstacles, such as the difficulty isolating the rare highly avid T cells that are specific for self-antigens expressed selectively or preferentially by tumor cells from most cancer patients; the requirement that transferred tumor-reactive T cells persist *in vivo*, traffic to tumor sites and function in an inhospitable immunosuppressive tumor microenvironment; and the potential for antigen or HLA loss tumor cells variants to escape recognition [35–38]. As a consequence, even when tumor-reactive T cells have been isolated and expanded from cancer patients, the adoptive transfer of these cells to treat malignancy was usually unsuccessful, and understanding the precise reasons for failure posed a formidable task. A proximal problem that was evident in the initial trials, and one that was distinct from the results of T cell therapy for viral infections after HCT was that the persistence of adoptively transferred tumor-specific T cells *in vivo* was remarkably short [6,8,9]. The basis for the differential persistence of adoptively transferred virus-specific T cells in HCT recipients and tumor-reactive T cells in cancer patients is now being revealed, and reflects both the environment into which the T cells are infused and

qualitative attributes of T cells that are isolated and expanded for adoptive transfer.

Depletion of endogenous lymphocytes to improve the efficacy of adoptively transferred tumor-specific T cells

The longest persistence of adoptively transferred T cells in humans was observed when virus-specific T cells were administered to immunodeficient allogeneic HCT recipients early post-transplant, when lymphopenia is typically present [3–5]. Although not initially recognized, a lymphopenic environment may contribute significantly to improving the persistence of transferred T cells by reducing competition for cytokines such as IL15 and IL7 that promote lymphocyte proliferation and survival; making 'space' available in the lymphoid compartment; and eliminating CD4⁺ CD25⁺ regulatory T cells and other cells with suppressor function [39–41]. Direct evidence that the induction of lymphopenia improves the persistence of transferred T cells was provided by studies from Rosenberg *et al.* in which melanoma patients were rendered lymphopenic either by treatment with chemotherapy alone or chemotherapy combined with total body irradiation, before the adoptive transfer of 10¹⁰–10¹¹ polyclonal melanoma-specific T cells [42,43,44^{••}]. In a significant subset of patients including those with advanced metastatic tumors, transferred T cells underwent dramatic *in vivo* expansion, persisted long-term, infiltrated into tumors and promoted tumor regression. As larger numbers of melanoma patients have been treated with T cell therapy following lymphodepletion, it has become clear that the improved antitumor activity that is observed correlates with better persistence of transferred T cells [45]. Studies in murine models have confirmed that lymphodepletion can be exploited to improve the antitumor efficacy of transferred effector T cells (T_E), and provided evidence that hematopoietic stem cell infusion with lymphodepletion further promotes the antitumor activity of T cell transfer [46].

It is now evident that inducing lymphopenia before adoptive T cell transfer improves the magnitude and duration of cell persistence, but additional studies are needed to determine the mechanisms involved and whether alternative strategies, such as administration of IL7 or IL15 combined with regimens that selectively or preferentially deplete regulatory or suppressor cells, might be equally effective with less toxicity. Additionally, the studies in melanoma have focused on infusing lymphocyte populations that predominantly contain cytolytic CD8⁺ T cells, and the contribution of tumor-specific CD4⁺ T cells to antitumor activity has not been extensively explored. A recent case report demonstrated that infusion of a CD4⁺ T cell clone specific for the NY-ESO TAA in a melanoma patient that did not receive lymphodepleting chemotherapy led to a durable remission, emphasizing that additional study of the CD4 subset is indicated [47[•]].

Isolating T cells for immunotherapy with the intrinsic capacity to persist *in vivo*

The quality of the T cells that are selected for expansion and adoptive transfer has been identified as an additional critical factor that determines the persistence of transferred effector cells. The T lymphocyte pool from which T cells for adoptive immunotherapy could potentially be isolated contains CD45RA⁺ CD62L⁺ naïve (T_N), CD45RO⁺ CD62L⁺ central memory (T_{CM}), and CD62L[−] effector memory (T_{EM}) subsets that differ in phenotype, function, and homing [48]. After recognition of antigen *in vivo*, T_N cells undergo proliferation and differentiation, resulting in the generation of large numbers of CD62L[−] effector T cells (T_E), most of which die as antigen is cleared leaving a small pool of T_{CM} and T_{EM} cells [49]. Memory T cells respond to antigen re-exposure *in vivo* and *in vitro* by differentiating again into T_E cells. The lifelong maintenance of T cell memory suggests that some cells in the memory pool may be capable of both self-renewal and differentiation, and there is evidence in mice that a subset of memory T cells may be endowed with stem cell like properties [50[•],51].

Despite the distinct attributes of T cell subsets, the origin of antigen-specific CD8⁺ or CD4⁺ T cells isolated for adoptive therapy has not been known with certainty in clinical trials. In the adoptive transfer studies for opportunistic viruses, T_E cells were obtained from donors with prior exposure to the pathogen and it is likely that the majority of the cells were derived from either T_{EM} or T_{CM}. In situations where tumor-reactive T_E cells are generated from the blood or tumor infiltrates of tumor bearing patients, the cells could be derived from T cells that have been exposed to TAA, but these cells are unlikely to have fully differentiated into memory cells in the presence of persistent antigen. Given the very low frequency of T cells specific for TAA in the blood of cancer patients, it is also possible that tumor-reactive T_E obtained after *in vitro* culture with antigen are derived from T_N precursors [52].

To provide insight into whether the origin of T_E cells influenced their ability to persist *in vivo* after adoptive transfer, we performed studies in non-human primates (*Macaca nemestrina*) to analyze the fate of CD8⁺ CMV-specific T_E cell clones derived from sort purified T_{CM} and T_{EM}. We selected non-human primates rather than mice because macaque and human T cells are similar in phenotype, function, and regulation, and culture conditions for propagating macaque T cells are identical to those for human T cell therapy. The cells were marked using retroviral vectors that encode with B-lineage molecules to enable tracking the transferred cells *in vivo*, expanded for at least 30 population doublings before adoptive transfer, and administered intravenously to animals with a full lymphoid compartment and without exogenous cytokines. T cell clones from both subsets had

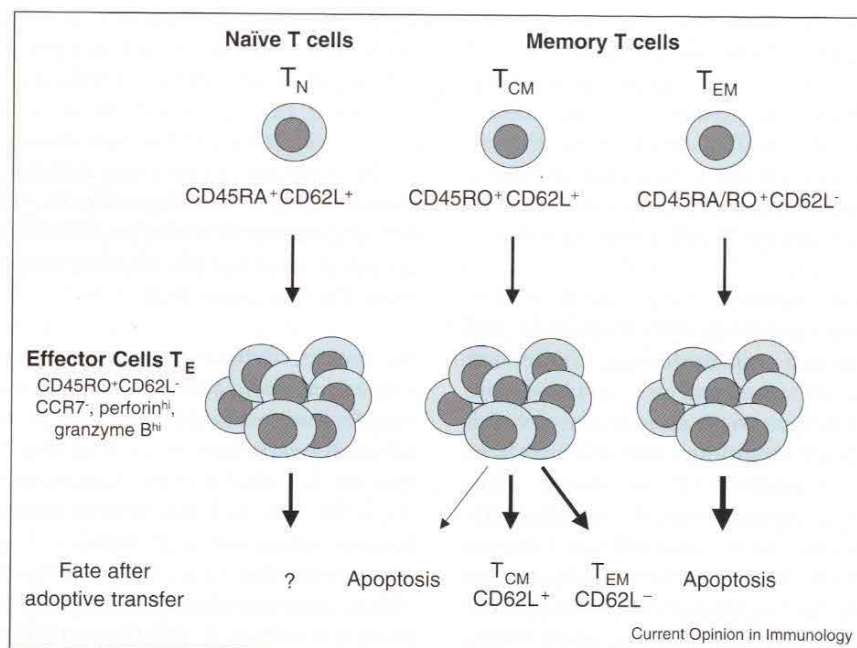
equivalent cytolytic and proliferative capacity, and uniformly expressed a T_E cell phenotype ($CD62L^-$ $CCR7^-$ $CD28^-$ $CD127^-$, granzyme B^{hi} and perforin ^{hi}). $CD8^+$ CMV-specific T_E clones derived from T_{EM} survived in the blood for only a short duration after adoptive transfer, failed to persist in lymph nodes (LN), bone marrow (BM), or peripheral tissues, and did not reacquire phenotypic markers of T_{CM} during their brief life span *in vivo*. By contrast, T_E clones derived from T_{CM} persisted in the blood long-term after adoptive transfer, migrated to memory T cell niches in the LN and BM, reacquired phenotypic properties of T_{CM} and T_{EM} , and responded to antigen challenge [53^{••}].

These results reveal profound differences in the survival of T_E derived from T_{CM} and T_{EM} after transfer into lymphoreplete hosts. T_E cells from T_{CM} have remarkable plasticity in the fates they adopt *in vivo*. Some of the T_E cells derived from T_{CM} reacquire $CD62L$ as well as other markers of T_{CM} and establish reservoirs of long-lived cells in lymph nodes while others adopt a $CD62L^-$ T_{EM} phenotype (Figure 2). Although T_E derived from T_{EM} have proliferative potential *in vitro*, these cells uniformly fail to survive *in vivo* after transfer into lymphoreplete hosts, suggesting that T_E derived from T_{EM} are incapable of reverting to the memory pool. It remains possible that lymphodepletion or exogenous cytokines will enable

T_{EM} -derived clones to persist, although these cells survive less well *in vitro* in IL-2, IL-7 and IL-15 compared to T_{CM} -derived T cells [53^{••}]. Additional studies are in progress to examine the fate of T_E derived from T_N precursors and to determine if the same results are obtained with $CD4^+$ T cells derived from T_{CM} and T_{EM} subsets. Murine studies using naïve TCR transgenic T cells would suggest that the culture conditions under which the T cells are primed is critical in determining the differentiation program of the cells [54,55].

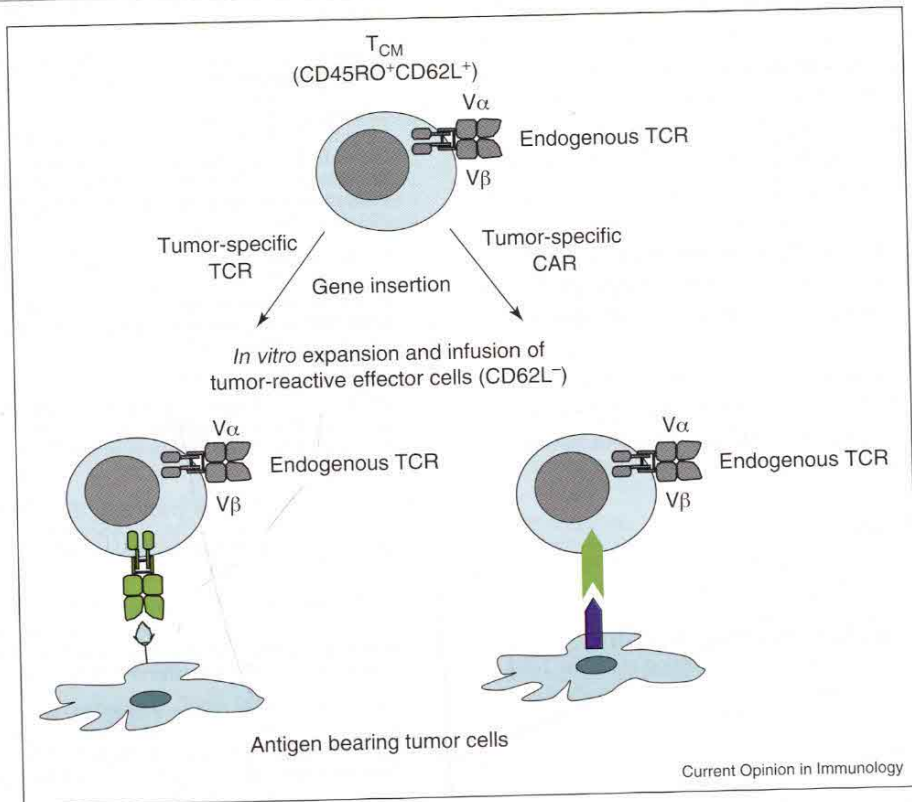
These results have implications for the types of T cells that should be selected for adoptive transfer, and for strategies to derive tumor-reactive T cells for immunotherapy of cancer. It is difficult and often impossible to detect or isolate T cells specific for TAA in blood or tumor infiltrating lymphocytes obtained from patients with solid tumors other than melanoma. However, gene expression profiling of tumors has identified many proteins that are aberrantly expressed or overexpressed in comparison with the normal tissue counterparts and might be targets for T cell therapy. Additionally, sequencing of the genomes of breast and colorectal cancer has identified an average of 90 nonsynonymous mutations, and *in silico* analysis suggests that many of these encode unique MHC binding epitopes [56,57]. The failure of the immune system to respond to a growing tumor in most

Figure 2



Fate of adoptively transferred $CD8^+$ effector T cells derived from distinct T cell subsets. Effector T cells (T_E) can be derived from naïve (T_N), central memory (T_{CM}), and effector memory (T_{EM}) subsets and express a $CD62L^-$, $CCR7^-$, granzyme B^{hi} , and perforin ^{hi} phenotype. Despite the similar phenotype at the time of adoptive transfer, T cells derived from T_{EM} precursors die rapidly after cell transfer and do not establish persistent T cell memory. T cells derived from T_{CM} precursors survive long-term *in vivo* and revert to both $CD62L^+$ and $CD62L^-$ memory cells. A subset of the transferred cells that re-express $CD62L$ and $CCR7$ reside in lymph nodes, downregulate expression of granzyme B and perforin and are capable of responding to antigen challenge. The fate of T_E derived from naïve precursors *in vitro* has not yet been definitively established in a large animal model.

Figure 3



Engineering tumor-reactive effector T cells by insertion of genes that encode tumor-specific T cell receptors or chimeric antigen receptors (CARs) into central memory T cells. The superior persistence of effector T cells derived from virus-specific T_{CM} suggests that this subset of T cells should be isolated based on their expression of CD45RO and CD62L for insertion of genes that encode TCR or CARs to target molecules on tumor cells. After insertion of the tumor-targeting receptors, the engineered T cells could be expanded *in vitro* and despite differentiating to cytolytic effector cells will retain the intrinsic capacity to persist *in vivo* after adoptive transfer and revert to the memory pool.

patients is due to evasion mechanisms including ignorance, local recruitment of regulatory T cells or other suppressor cells, and expression or secretion of inhibitory molecules or cytokines that impair responses locally and/or systemically. However, T cells specific for TAA can be derived by *in vitro* priming from the naïve repertoire of normal individuals and the T cell receptor (TCR) alpha and beta genes isolated from these T cells and inserted into T cells from the cancer patient to confer a desired specificity for an MHC/peptide complex [58^{••},59]. Indeed, many groups are now assembling libraries of TCRs that target human TAA and could be used to engineer tumor-reactive T cells for therapy. In a similar fashion, chimeric antigen receptors (CARs) constructed by fusing single chain antibody domains to the TCR ζ chain alone or in combination with costimulatory signaling domains, can be introduced into T cells to target surface molecules expressed on tumor cells and overcome the requirement for MHC restriction [60]. The demonstration of the superior engraftment properties of T_E derived from T_{CM} would suggest that selection or enrichment of T_{CM} before insertion of tumor targeting receptors will provide a superior T cell product for adoptive

therapy, and may overcome the inconsistent cell persistence observed in initial studies [58^{••},61] (Figure 3). The results of a recent study of T cell therapy for neuroblastoma support this hypothesis. T_E cells derived from cultures of EBV-specific T_E cells and engineered with a CAR specific for the diasialoganglioside GD2 on neuroblastoma exhibited superior persistence compared with unselected T cells activated with anti CD3 monoclonal antibody and engineered with the identical CAR [62[•]]. The results suggest that intrinsic programming of the EBV-specific T cells and/or recognition of antigen through the endogenous TCR were important for *in vivo* persistence of the tumor-reactive T cells.

Conclusions

Substantial progress has been made in understanding the requirements to effectively utilize adoptive T cell therapy for human viral infections and melanoma. The encouraging results now being achieved in small clinical trials have diminished previous pessimism, and the field appears poised to apply adoptive therapy more broadly to human malignancies. However, the techniques required for this endeavor remain complex and involve isolating or

engineering by gene transfer T cells that recognize malignant cells, expanding the tumor-reactive cells *in vitro*, and conditioning the host to promote the survival and function of transferred T cells. Malignancies are formidable adversaries and employ many strategies to overcome immune recognition, which may include defects in the processing or presentation of TAA, recruitment of suppressor cells, and production of factors that disable tumor-reactive T cells. Obtaining T cells with the capacity to persist *in vivo* and achieving a quantitatively large tumor-reactive T cell response by conditioning the host before adoptive transfer represent key steps in improving the efficacy of adoptive T cell transfer. It will be essential that future clinical trials evaluate the characteristics of tumors that fail to respond to therapy to derive insights into the properties of tumor cells that limit therapeutic efficacy, and lead to the rationale modifications to improve efficacy.

Acknowledgements

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