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Re-adapting T cells for cancer therapy: from mouse models to clinical trials

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Summary

Adoptive T-cell therapy involves the isolation, expansion and reinfusion of T lymphocytes with a defined specificity and function as a means to eradicate cancer. Our research has focused on specifying the requirements for tumor eradication with antigen-specific T cells and T cells transduced to express a defined T-cell receptor (TCR) in mouse models and then translating these strategies to clinical trials. Our design of T-cell-based therapy for cancer has reflected efforts to identify the obstacles that limit sustained effector T-cell activity in mice and humans, design approaches to enhance T-cell persistence, develop methods to increase TCR affinity/T-cell functional avidity, and pursue strategies to overcome tolerance and immunosuppression. With the advent of genetic engineering, a highly functional population of T cells can now be rapidly generated and tailored for the targeted malignancy. Preclinical studies in faithful and informative mouse models, in concert with knowledge gained from analyses of successes and limitations in clinical trials, are shaping how we continue to develop, refine, and broaden the applicability of this approach for cancer therapy.

Keywords

adoptive T-cell therapy; TCR gene therapy; high affinity; preclinical models; clinical trials

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Introduction

Although the question of whether the immune system plays an active role in protecting individuals from the development and progression of tumors was long debated (1), there is now abundant and unequivocal evidence that many components of the immune system can effectively eliminate nascent tumor cells and prevent the outgrowth of tumors (1, 2). However, the vast majority of malignancies appear to evade or subvert the immune system, and endogenous T-cell responses are generally inadequate to eradicate established tumors. Consequently, strategies to reproducibly harness the immune system for eradication of a tumor after it has become established have proven more elusive. Effector responses mediated by T cells appear best suited to this task. For example, it has long been known that donor-derived T cells can target and eliminate leukemic cells following allogeneic hematopoietic cell transplant (HCT) (3), and T-cell-derived effector molecules including IFN γ (4) and perforin (5–7) contribute to the inhibition of the development of chemically or virus-induced tumors. Adoptive T-cell therapy, developed as a means to selectively amplify a potentially effective antitumor T-cell response *in vivo*, has proven efficacious for the treatment of some patients with established tumors. Although substantial success has been achieved in melanoma (8), for most other tumors only modest therapeutic benefit has been observed, due in large part to challenges associated with generating and/or sustaining a potent immune response targeting the tumor. We have endeavored to dissect and address these challenges through the use of animal models that can inform clinical studies for the treatment of human malignancies. In the sections that follow, we will elaborate on our efforts to clinically translate the therapeutic administration of *ex vivo* expanded T cells that either naturally express or have been genetically engineered to express a tumor antigen-specific TCR, discuss strategies we are pursuing to generate and assess the safety of enhanced-affinity TCRs, and outline our recent studies designed to adapt these approaches to effectively eliminate solid tumors.

Immune tolerance presents a major barrier to achieving effective anti-tumor immunity

A variety of immune tolerance mechanisms exist that limit the erroneous activation of T cells specific to self-antigens that might cause autoimmunity, but these same mechanisms can also decrease the efficacy of endogenous antitumor immune responses. The limited efficacy of therapeutic vaccines in patients who already have detectable tumors in large part reflects the induction of at best weak responses. This is due not only to the poor immunogenicity of most of the vaccine regimens tested to date and the compromised patient immune systems but also to central and peripheral tolerance mechanisms that are operative (9). The first barrier that limits endogenous T-cell recognition of self/tumor antigens occurs during T-cell development. $\alpha\beta$ T-cell development in the thymus involves a sequential process of *Tcrb* and *Tcra* gene rearrangements, followed by a series of TCR-mediated selection events. Progenitor thymocytes must express a TCR that has some minimal affinity for self-peptide in an MHC molecule (pMHC) for positive selection, but T cells expressing TCRs that strongly recognize self-pMHC undergo programmed cell death (negative selection). High affinity T cells specific for candidate tumor antigens that are non-mutated

self-antigens are likely candidates for such negative selection. It has been estimated that negative selection reduces the TCR affinity of peripheral self/tumor-reactive T cells ~1.5 logs (10, 11).

T cells that express self/tumor-reactive TCRs do exist in the periphery of normal individuals, albeit with sub-optimal TCR affinity, and such endogenous potential antitumor responses are further hampered by peripheral tolerance mechanisms, including anergy, ignorance and active suppression by CD4⁺FoxP3⁺ Tregs. These mechanisms present additional barriers to achieving effective T-cell-based therapies for cancer. Strategies to bypass such tolerance mechanisms when targeting self/tumor antigens pose a risk for toxicity due to T-cell recognition of low levels of self-antigen on normal tissue. Using a model in which the tumor antigen was engineered to also be expressed by a normal tissue (12, 13), we found that T-cell tolerance could be overcome and function rescued to achieve tumor eradication by initially employing various means to induce tolerant T cells to proliferate independent of signals delivered through the self-reactive TCR (14–16). Surprisingly, T-cell-mediated autoimmunity was not observed, indicating that there are settings in which tolerant T cells can be functionally rescued and the now responsive T cells can be safely and effectively employed in adoptive therapy to mediate antitumor activity. Thus, defining the principles for identifying/generating and harnessing such responses has become a major focus of our studies.

Identification of candidate target tumor antigens

Malignant cells originally derive from normal self-tissue and thus in part are able to proliferate and progress because the immune system has difficulty distinguishing the tumor cells from self (17). A fundamental goal of adoptive T-cell therapy is to isolate or generate T cells that will destroy tumor cells while ignoring normal self-tissue. Therefore, the identification of appropriate tumor antigens to target has been the subject of intense research (18). The most promising target antigens would be those not expressed by normal self-tissues to limit the risk of toxicity, uniformly expressed at high levels by the malignancy in most individuals to provide a commonly useful target, and that contribute to the malignant phenotype to limit the potential for escape. Tumor-specific mutations can generate new T-cell epitopes that are unique to the tumor and can be targeted by T cells. Well-defined oncogenic fusion proteins such as *TEL-AML1* or *BCR-abl* fusions have thus been considered attractive targets, but T-cell responses to such antigens have been hampered by limited processing of the protein and/or the presence of only a few unique epitopes that can bind to HLA alleles (19, 20). Likewise, tumors that are induced by oncoviruses may express foreign viral proteins that can be targeted by T cells. Although these antigens represent promising targets in virus-induced malignancies, many of the candidate viral proteins, such as those expressed in EBV- and HPV-associated malignancies, have thus far been disappointingly poorly immunogenic (21, 22).

However, most malignancies lack obvious non-self-antigens to target, and for these cancers effective anti-tumor T-cell therapy will likely require the identification of ectopic, aberrant, or over-expressed self-antigens. Promising clinical responses have been reported targeting metastatic melanoma with overexpressed melanoma self/tumor antigens such as gp100 and

Melan-A/MART-1 (23–26). Other promising targets include oncofetal antigens that are expressed in abundance in normal cells only during fetal development but are ectopically expressed in tumors. Many of these proteins have been associated with proliferative and/or survival advantages, and thus can promote the malignant phenotype. WT1 is an antigen detected at high levels in many malignancies, including most AML, MDS, and CML cases, with higher levels correlating with worse prognosis (27–29). WT1 is highly expressed during embryogenesis, when it is essential for development of the urogenital tract, but after birth normal expression is limited to low levels in a few cell types, including kidney podocytes, and CD34⁺ hematopoietic stem cells (HSCs) (30, 31). Leukemic cells express 10–1000x more WT1 than normal CD34⁺ HSCs (32, 33), with leukemic ‘stem cells’ expressing the highest levels (34). We are currently pursuing strategies to target WT1 with adoptively transferred T cells expressing endogenous or transgene encoded TCRs with a high affinity for the WT1 antigen. Another category of antigens are termed cancer-testis antigens, which are characterized by normal adult expression that is restricted to germ cells such as those found in the testis and ovary. The function of many of these cancer-testis antigens are poorly defined, though some appear to have important functions in meiosis that may be co-opted to promote oncogenesis (35, 36). Cancer-testis antigens, such as NY-ESO-1 and MAGE antigens, can be highly expressed in tumor cells and thus represent attractive and likely safe targets for adoptive T-cell therapy (37).

An alternative approach is to target a highly expressed tissue-restricted antigen expressed only by the tumor’s tissue of origin. As this can result in T-cell-mediated destruction of both normal and malignant cells of the target cell lineage, for this approach to be viable the antigen must be expressed only in the tissue of origin, and that tissue must not be necessary for life. For example, this strategy has been pursued in melanoma by targeting melanosomal proteins that can result in induction of vitiligo as a result of destruction of normal melanocytes (38, 39). More recently, dramatic effects against B cell leukemias have been observed with T cells genetically modified to express chimeric antigen receptors (CARs) (40, 41), which contain immunoglobulin ectodomains for recognizing the targeted antigen that have been fused to signaling components of the TCR complex (CD3 ζ) as well as the intracellular signaling domains of costimulatory molecules such as CD28 and 41BB. Abs have substantially higher affinities for their targeted antigen than TCRs have for pMHC, which has advantages and disadvantages when used as a recognition structure in T cells, and CARs specific for a B-cell lineage-restricted surface molecule such as CD19 have been reported to efficiently target and eliminate both malignant and normal B cells (40–42), as discussed in more detail elsewhere in this issue. Mesothelin is another promising differentiation antigen, with expression in normal human tissues restricted to low levels in the mesothelial lining of the pleura, pericardium and peritoneum (43), but much higher level expression that contributes to tumor invasiveness (44, 45) detected in many human cancers including mesothelioma and adenocarcinomas of the pancreas and ovary (46, 47). We are developing strategies to target these solid tumors with adoptively transferred T cells expressing high affinity Mesothelin-specific TCRs, and will describe some of our studies in a mouse autochthonous tumor model to assess the efficacy of this approach.

Bench to bedside: proof of concept and refinement

The requirements necessary for tumor eradication were initially established in mice that had been injected with the Friend retrovirus (FV)-induced erythroleukemia (FBL)(48, 49). In this model, immunocompetent adult mice succumbed to the rapidly disseminating leukemia, which models the events in acute leukemia in humans. However, tumor-bearing mice treated with a short course of non-myeloablative chemotherapy were able to eradicate their leukemia if they then received T cells from mice that had been immunized with irradiated FBL cells (48). To mediate tumor eradication the T cells had to be specific for the tumor (50), capable of proliferation in the host (51) and able to persist *in vivo* for a prolonged period after transfer (48, 52). These early studies provided a foundation for translating adoptive immunotherapy to humans, and specified that adoptively transferred cells would require establishing a population of tumor antigen-specific T cells capable of persisting and functioning long term *in vivo* after transfer to obtain the desired therapeutic benefit.

We have initially explored clinically adoptive transfer strategies aimed at increasing the number and quality of either autologous T cells targeting melanoma-associated antigens or T cells derived from an HLA-matched allogeneic hematopoietic cell donor that recognize the leukemia associated antigen WT1 for use as therapy in the post-transplant setting (23, 26, 53–55). In these first generation studies in leukemia, *ex vivo*-expanded tumor-specific CD8⁺ T cells were generated by first stimulating T cells with autologous peptide-pulsed dendritic cells followed by cloning the responding T cells via limiting dilution. The CD8⁺ T-cell clones that expanded and exhibited the greatest avidity/cytolytic activity were then selected and further expanded for infusion. This strategy of generating cloned T cells allowed us to administer a homogenous population of antigen-specific cells that could be precisely characterized, thus reproducing the primary goal of therapeutic vaccination by increasing frequencies of tumor-reactive T-cells as well as providing a setting in which the fate of the elicited response can be tracked (56).

Although the infusion of these cell products exhibited a very tolerable safety profile, efficacy in reducing established tumor burdens was observed in only a limited number of patients (23, 26, 55, 57). The major obstacle encountered was the short persistence of T-cells *in vivo* after infusion, which correlated with the absence of sustained clinical responses. Conditioning regimens with chemotherapy (high-dose CY and fludarabine) that are transiently cyto-reductive or with total body irradiation (1200 rads TBI) that is myeloablative administered before infusions of tumor-infiltrating lymphocytes (TILs) have been shown by the Rosenberg group to facilitate the *in vivo* engraftment and expansion of adoptively transferred cells in the lymphodepleted hosts, but at the cost of increased toxicity (8, 58, 59). However, more recently regimens using less intense conditioning or no conditioning prior to adoptively transferred cells have shown that myelo-ablation is not an absolute requirement to establish long-term T-cell persistence (23, 40, 41, 53, 60). In the studies from our group using clonal T cells, cytoreductive chemotherapy before the T-cell infusion only partially improved persistence (23, 55). Similarly, following the cell infusions with administration of low-doses or high-doses of the pro-proliferative and pro-survival cytokine IL-2 did not have a significant differential effect on long-term persistence (23, 26, 55). Thus, the absence of persistent T cells in the majority of patients participating in these

studies may have reflected the nature of the infused cells, which had been extensively expanded from a single reactive cell, and thus may have been driven to terminal differentiation *in vitro* with consequently very limited/absent remaining replication potential after transfer. Therefore, although current cytoreductive regimens and cytokine infusions may enhance the survival of transferred T cells that otherwise compete poorly with endogenous T cells, the necessity for toxic pre-conditioning of the host to facilitate engraftment may become obsolete with continuous improvements in T-cell generation that improve the quality of the T-cell infused.

Despite the poor persistence of transferred tumor-specific T-cell clones in most patients, phenotypic and functional analysis of T cells before and after infusion from the subset of patients in whom persistence of the transferred T cells was observed has provided insights into desirable cell intrinsic characteristics. Following treatment of either melanoma or leukemia patients, we repeatedly observed that T cells that did persist had either maintained characteristics associated with long-lived memory cells through the *in vitro* culture period or acquired those characteristics *in vivo* after transfer (23, 53). These included several key traits suggestive of an enhanced ability to proliferate as reflected by detecting Ki-67 expression *in vivo*, including expression of co-stimulatory molecules (CD27 and CD28) and receptors associated with survival signals (CD127)(61–63), ability to secrete the autocrine growth factor IL-2 in response to cognate antigen (64), and ability to localize to lymphoid-associated tissue (CD62L, CCR7) (65). These observations are reminiscent of studies with murine LCMV infection, which revealed that transferred CD8⁺ T cells derived from the central memory T-cell pool (Tcm) provided enhanced protective immunity from *in vivo* challenge compared to cells derived from the effector memory T-cell pool (Tem)(66), as well as studies in non-human primates that demonstrated enhanced persistence of *in vitro* expanded CD8⁺ T cells derived from Tcm versus Tem (67). However, in the patients in which long-term persistence was achieved, the recovered transferred cells possessing such characteristics were detected mostly in the absence of detectable disease burden, either due to tumor regression following adoptive T-cell transfer or because patients received the cells at a time of having minimal residual disease or prophylactically without detectable disease at the time of infusion. This outcome is consistent with evidence that formation of CD8⁺ Tcm after viral infection occurs only if the antigen is cleared (68). Therefore it remains unclear if it is the disappearance/absence of antigen or the type of cells infused (or both) that allowed maintenance/acquisition of these characteristics, but identifying strategies that permit establishment of a long term persisting T-cell response remains a desirable goal to achieve.

One strategy for infusing cells products with increased capacity to persist after transfer is to infuse cells that have undergone limited proliferation and expansion *in vitro*. This has become more feasible with the current availability of clinical grade high-speed sorters that use wholly interchangeable tubing sets to avoid contamination between products. Clinical-grade cell products can be generated by expanding tumor-specific T-cell lines with antigen *in vitro*, the desired antigen-specific population selected with specific pMHC multimers, and the polyclonal cells then expanded for 1 or 2 cycles of stimulation before infusion. These antigen-specific polyclonal cell products bypass the limiting dilution cloning process which reduces *ex vivo* production times from >3 months to ~4–6 weeks and results in less

terminally differentiated products in which each cell has undergone far fewer divisions. However, the products are consequently less homogenous than T cell clones and contain antigen-specific cells of multiple avidities that are less readily tracked *in vivo* since quantitative PCR for a single rearranged TCR gene cannot be employed. This strategy is being applied by our group to generate antigen-specific cells for current clinical trials, and thus far all infused products have remained detectable *in vivo* for >60 days.

Modifying culture conditions to generate antigen-specific T-cell products with improved persistence

Endogenous memory responses to tumor-associated antigens are rare/non-existent in the peripheral blood of most individuals with cancer and usually absent in healthy individuals. In an effort to establish T-cell-mediated anti-leukemic activity for patients with a high-risk of or with already relapsed leukemia after HCT without the risk of accompanying GVHD, we performed a clinical trial in which WT1-specific CD8⁺ T-cell clones were generated from the normal repertoire of each patient's HLA-matched donor and transferred after HCT (53). No *de novo* WT1-specific T cells could be detected *ex vivo* by tetramer staining of PBL from >37 healthy donors analyzed, and thus the CD8⁺ T cell clones generated and expanded for infusions had to be derived from the naive repertoire. We have previously shown that CD8⁺ T cells to a selected antigen can be primed, expanded, and cloned from the naive repertoire using mature, activated DCs as the antigen-presenting cell (APC) to initiate the response (54). The resulting *in vitro* expanded CD45RO⁺ T cells expressed a fully differentiated effector phenotype, but lacked expression of molecules associated with enhanced survival (CD28 or CD127) or lymph-node homing (CD62L, CCR7) (53). Furthermore, T cells obtained using this technique transferred into leukemia patients with or without detectable disease exhibited only transient *in vivo* survival (14 days) and stopped proliferating shortly after transfer (53).

During the course of this clinical trial, studies evaluating the biology and activity of interleukin (IL)-21, a γ_c receptor cytokine family member, were providing increasing evidence that IL-21 not only promotes CD8⁺ T-cell proliferation similar to other γ_c cytokines, but also is critical for the formation of memory rather than differentiated effector cells following antigen stimulation (69–72). When used with IL-15 during the priming of both murine and human antigen-specific CD8⁺ T cells, IL-21 was shown to promote greater expansion, prevent apoptosis of the cells responding to antigen stimulation, and lead to *in vitro* generation of CD8⁺ T cells with a CD28^{hi}, less terminally differentiated phenotype (70, 71, 73). Furthermore, murine naive CD8⁺ T cells primed in the presence of IL-21 exhibited enhanced *in vivo* persistence after transfer (74). Thus, exposure to IL-21 during priming appears to induce the responding CD8⁺ T cells to express a more central memory-like program. Based on these encouraging results, GMP-grade IL-21 was produced and made available for incorporation into the cell culture process during the priming of antigen-specific T-cells for use in clinical trials.

We found that WT1-specific CTL clones that had been primed in the presence of IL-21 before cloning expressed significantly higher levels of CD27, CD28 and CD127 after expansion and prior to infusions, consistent with a less differentiated and more central

memory-like phenotype, despite the extensive expansion *in vitro*. These CTL clones also exhibited enhanced *in vivo* persistence and proliferation after transfer as compared to CTL clones generated without exposure to IL-21. Furthermore, as previously shown in a murine model of LCMV infection, the persisting cells generated with exposure to IL-21 displayed phenotypic and functional characteristics of T_{cm} (69). Thus, naive cells exposed to IL-21, albeit for only a very limited time during the early priming phase of the *in vitro* generation process, give rise to antigen-experienced T cells with less terminally differentiated, more T_{cm}-like characteristics *in vitro* that can persist for prolonged periods of time *in vivo* after adoptive transfer.

Modifying the host environment with checkpoint blockade reagents to improve the anti-tumor efficacy of adoptively transferred cells

Another strategy being pursued to increase the efficacy of transferred T cells is to administer checkpoint blockade inhibitors after T-cell transfer. Abs to CTLA-4 have been shown to block the interaction between CTLA-4 and the costimulatory molecules B7.1 and B7.2 (CD80 and CD86), thus removing the CTLA-4 inhibitory signal which can follow antigen-specific engagement of the TCR. The critical negative regulatory role resulting from signaling via CTLA-4 was established in mice genetically engineered to knock-out the CTLA-4 gene, which exhibited hyperproliferation of lymphocytes, diffuse lymphadenopathy, and early death (75, 76). A mAb to CTLA-4 (ipilimumab, Yervoy) was approved by the FDA for use in patients with unresectable melanoma in April 2011, after landmark studies demonstrated an overall response rate of >70% in patients who had received ipilimumab alone, or in combination with a peptide vaccine (77, 78). However, the number of patients who experienced prolonged responses was <10%, suggesting the magnitude, breadth, and/or maintenance of responses triggered *de novo* from the naive T-cell repertoire or activated from pre-existing antigen-experienced melanoma-reactive T cells by anti-CTLA4 alone is not sufficient to eradicate the tumor in most cases. Combining antigen-specific T cell transfer with checkpoint blockade can potentially serve a double purpose. Firstly, providing patients with a source of tumor-reactive T cells that express detectable levels of CD28 that can interact unopposed with CD80 and CD86 in the presence of CTLA4 blockade, could allow the expansion/selection of an antigen-specific population with the capacity to benefit from autocrine IL-2 secretion. This may in turn potentially prolong *in vivo* survival of the transferred T cells. Secondly, the administration of antigen-specific cells with enhanced survival properties may provide greater anti-tumor activity directly as well as indirectly by facilitating the release of tumor antigens from lysed tumor cells in a pro-inflammatory environment. The tumor antigens released may trigger in the presence of anti-CTLA4 the *de novo* generation of new anti-tumor specific responses to non-targeted antigens (79). Thus, the combination may represent a strategy to increase the number and strength of T cells targeting multiple antigens specific to the patient's own tumor. A clinical trial being performed in collaboration with Cassian Yee has been currently enrolling patients with metastatic melanoma to address this hypothesis.

Administration of antigen-specific cells in the context of extensive tumor burdens can lead to 'exhaustion' of the cells *in vivo* after transfer, and another promising approach is to

combine T-cell transfer with immune-modulating reagents that block the inhibitory PD1/PDL-1 axis as a means to dampen the loss of function that can be triggered by the binding of PD1 on the surface of T cells with its ligand PD-L1 expressed on tumor cells (80). Blocking the PD-1/PD-L1 axis has been shown in early clinical trials to result in durable responses in a fraction of patients with melanoma, renal cell carcinoma and non-small cell lung cancer (81, 82). These encouraging results have both further demonstrated the potential for T cells to eradicate tumors and highlighted that in most patients the endogenous response elicited by the tumor is either not broad enough or of sufficient magnitude to eradicate the tumor. However, combining PD1/PDL-1 blocking agents with the transfer of large numbers of tumor-reactive T cells may provide an opportunity to achieve potent and sustainable anti-tumor responses *in vivo*, and we are currently planning a trial to assess this possibility.

Bedside back to bench: preclinical modeling to improve efficacy

When generating HLA A*0201-restricted CD8⁺ T-cell clones targeting WT1 for patients with high risk leukemia in the post-HCT setting in our previous trial (53), a large panel of CTL clones were obtained for each patient-donor pair, and the most avid clone that could be expanded was selected for infusion based on the ability to lyse targets pulsed with titrating amounts of the peptide epitope. However, direct comparisons of the most avid WT1-specific CTL clones obtained from each healthy donor demonstrated a wide variety of functional avidities, with the peptide concentration required to pulse HLA-A2⁺ target cells to achieve 50% target lysis ranging over 4 logs, from 10⁻¹ to <10⁻⁴ ng/ml (Fig. 1A). As WT1 expression after birth is present only at low levels in limited tissues, including kidney podocytes and CD34⁺ HSCs (83–85), on-target off-tissue toxicities were not observed even in patients who had received the clones with comparatively higher avidities that persisted at detectable levels for up to 14 months (53). The infusions were well tolerated, and new onset GVHD was also not observed. Importantly, two patients who had received clones with higher avidities and had detectable disease exhibited direct evidence of antileukemic activity. One patient with detectable leukemic blasts in the blood had a rapid reduction in the percentage of blasts after the CTL infusion, becoming 0% of total WBCs after 14 days, which correlated with the presence of the infused WT1-specific CTL in the blood (Fig. 1B). Unfortunately, the patient developed a toxicity from the extensive prior therapy that precluded further T-cell infusions, and as the cloned CTL, which had been generated before IL-21 was used during priming, disappeared from the periphery, his leukemic blasts re-appeared. The second patient cleared minimal residual disease (MRD) that was detected by flow cytometry and PCR in the bone marrow prior to the CTL infusions. This patient did receive cloned CTL primed in the presence of IL-21, and after infusions these cells peaked with frequencies up to 40% and persisted in the blood for at least 10 months at frequencies of ~6% (Fig. 1C). Although the results from this trial were encouraging and suggested that the infusion of T cells with high avidity was safe and can mediate antileukemic activity, the data also highlighted the importance of generating high avidity T cells that can persist after transfer for all patients.

Adoptive T-cell therapy in the era of gene therapy

TCR gene therapy is an emerging strategy that has several benefits over conventional adoptive T-cell therapy. As discussed above, the efficacy of adoptive T-cell transfer in previous clinical studies correlated with the quality of the infused T cells, which appeared to be at least in part dependent on the differentiation state of the original *ex vivo* generated antigen-specific clone. With TCR gene therapy, a defined T-cell lineage can be selected for transduction that has desirable qualities, such as the ability of central memory cells to self-renew and persist better *in vivo*, functional characteristics such as cytolytic activity and cytokine production, and/or the capacity to resist negative regulation. Furthermore, trials can be designed so that all patients are treated with T cells expressing an identical high affinity TCR, which will have been thoroughly characterized for both safety and antitumor efficacy prior to treatment. Thus it is possible to achieve a level of standardization and reproducibility, as well as quality, that is not possible with patient-specific clones or sorted polyclonal T-cell populations.

Choice of substrate cell

The particular T-cell lineage(s) that would represent the most effective substrate for TCR gene therapy have not been clearly defined, and it is likely that this may vary with the tumor setting and tumor burden being treated. Previous studies in mice and humans have shown that *in vivo* persistence of transferred T cells is a necessary prerequisite for an anti-tumor response (23, 86), and we have found that both *in vivo* persistence and antitumor activity were associated with the acquisition of Tcm phenotype in patients that received *in vitro* expanded tumor-reactive T cells. Therefore, we have focused on T cells with a central memory phenotype as a cell lineage that would be desirable for TCR gene therapy. This hypothesis is supported by a study from the Riddell lab in non-human primates in which T-cell clones derived from the CD8⁺ Tcm pool exhibited much greater replicative potential in response to antigen and much greater persistence following transfer than T-cell clones derived from a CD8⁺ Tem population, despite all infused cells identically displaying a differentiated Tem phenotype after *in vitro* expansion and before infusion (67), suggesting that cells in the Tcm lineage have been imprinted with qualities that facilitate *in vivo* survival. Moreover, many of the phenotypic effector cells derived from the Tcm lineage were capable of reverting back to Tcm cells *in vivo*, providing more evidence of epigenetic imprinting of these cells. A subsequent study in mice in the setting of established tumor found that naive T cells mediated enhanced cytokine production and greater antitumor activity than Tcm cells (87). Thus, the 'ideal' cell to transduce for therapy may well be context dependent. However, the choice may also be restricted by other considerations. For example, for treatment of patients post-HCT, the use of donor-derived naive T cells rather than memory cells would pose an increased risk of GVHD, as donor naive T cells contribute disproportionately to GVHD following HCT (88, 89). The more diverse TCR gene repertoire of the naive pool would increase the risk of mispairing that can occur between endogenous and transgene-encoded TCR chains and form an autoreactive receptor (see below). Thus, we have been focusing for treatment of leukemia in patients post-HCT on Tcm-derived donor cells, but for solid tumors or settings of large persistent antigen burdens multiple infusions of effector T cells or use of transduced naive T cells should be evaluated (90).

Vector design for TCR gene therapy

Although the ectopic expression of a second, tumor-reactive TCR on the surface of a transduced T cell appeared to be a fairly straight forward endeavor, it took at least a decade of research to overcome the various challenges of this approach to create reagents that can make this therapy more reliably useful in the clinic. T cells generally express only a single functional TCR $\alpha\beta$ pair, which is expressed on the T-cell surface in a complex with CD3 ϵ , CD3 γ , CD3 δ , and CD3 ξ (91). This multi-chain organization of the TCR complex introduces several obstacles to efficient expression of a therapeutic TCR transgene. Because TCR surface expression is limited by the availability of the CD3 components, the avidity of the transduced T cell for the tumor will depend on how effectively the introduced TCR chains out-compete the endogenous TCR chains for surface expression in association with the intact CD3 complex. The first issue is designing a TCR expression construct that ensures coordinated, high level expression of both TCR chains. High-level expression requires a strong promoter to drive transgene expression (92, 93), and codon optimization of the TCR genes to increase the efficiency of protein translation (94). Coordinated expression of the TCR α and TCR β chains to maximize appropriate pairing can be accomplished by expressing both chains as a single transcript, separated by a 'self-cleaving' picornavirus-derived 2A element (95, 96). These modifications appear to generally provide advantages for expression of the introduced TCR chains compared to the endogenous TCR chains, but some paired TCR α and β chains appear to intrinsically associate more efficiently with CD3 components, and such "strong" TCRs can naturally outcompete most endogenous receptors for surface expression. As the qualities of a TCR $\alpha\beta$ pair that make it a strong TCR have not been defined, identifying strong TCRs for use in gene therapy requires screening for efficient surface expression of the introduced TCR.

The competition for surface expression is more than just between endogenous and introduced TCR chains. because there is a possibility of mispairing of the α and β chains from the introduced TCR with respectively the β and α chains of the endogenous TCR, leading to the expression of two new TCRs with unknown, potentially auto-reactive antigen specificities (97, 98). We have shown that mispairing with endogenous TCR chains can be very significantly diminished by introducing point mutations into the TCR chains creating a second cysteine in the external membrane proximal region of the TCR α and TCR β constant domains, which promotes preferential pairing between the introduced TCR chains. Additionally, this appears to enhance association with the CD3 complex, presumably by facilitating more efficient assembly in the ER, and leads to increased surface expression of the introduced TCR (and reduced expression of any other paired TCR chains), thereby further creating a 'strong' TCR (99).

WT1-specific TCR gene therapy trial

To translate these insights to the clinic, we devoted considerable effort to identify and characterize a high affinity WT1-specific TCR suitable for introduction and expression into T cells that would impart high avidity for the WT1-expressing target. The functional avidity of the transduced T cell, which reflects the overall strength of binding of the effector and target cell and determines the sensitivity of triggering and ability to mediate effector

functions, is in large part a consequence of the affinity of the TCR, but is also impacted by the expression/type of co-stimulatory adhesion molecules expressed by the T cell and the ligands expressed by the target. Thus, >1000 clones from >70 HLA-A*0201⁺ normal donors were screened to identify CTL clones with high avidities, and we selected the clone (denoted clone C4) containing the TCR that appeared to have the highest affinity based on the ability to bind the lowest concentration of WT1/HLA A*0201 pMHC multimer. To minimize the potential risk of on-target off-tissue toxicity, the selected C4 TCR was purposefully isolated from the peripheral repertoire of a healthy HLA*0201⁺ donor, guaranteeing that the TCR had undergone negative selection during thymic development, and that the T cells expressing the TCR were not mediating an autoimmune process in the periphery. The selected TCR was inserted into a third-generation lentivirus construct for expression in human cells, and codon-optimized to achieve high-level protein expression. The final construct was engineered to be encoded as a single open reading frame consisting of the TCR β and TCR α chains separated by a 2A element from the porcine teschovirus (P2A) to ensure coordinated gene expression (95, 96), and incorporated complementary cysteine residues by introducing point mutations in the constant domains of the TCR α and TCR β genes to promote inter-chain pairing and discourage mispairing of the introduced TCR chains with endogenous TCR chains (99).

The safety and efficacy of this characterized TCR introduced into HLA-A*0201-restricted EBV- or CMV-specific donor cells is currently being tested in a phase I/II trial for patients with high-risk leukemias who have undergone HCT and are at a high risk of disease recurrence (100). The use of virus-specific (preferably EBV or if necessary CMV) cells as the cell substrate for expressing the characterized WT1-specific TCR in the post-HCT setting serves three purposes: (i) this restricts the antigen specificity of the endogenous TCRs expressed by the substrate cells, decreasing the likelihood of transducing T cells that will recognize host self-antigens; (ii) this ensures that the transduced T cells will be in large part derived from a central memory T-cell pool and therefore more likely to be endowed with properties that enhance *in vivo* persistence; and (iii) since the transduced cells can still respond to viral antigens, survival and function may also be prolonged *in vivo* by intermittent signaling through the endogenous virus-specific TCR. Thus far, only a very limited number of patients have been treated, but no on-target off-tissue toxicities or new-onset GVHD have occurred to date, suggesting this approach using allogeneic donor T cells appears safe. Moreover, since WT1 over-expression is not restricted to leukemias but rather is present in many tumor types (101), such TCR gene therapy should be readily adaptable to transducing autologous memory or naive T cells primed in the presence of IL-21 for patients with solid tumors. Indeed, in concert with Dr. Stan Riddell's group, we will soon be initiating a trial in patients with non-small cell lung cancer, in which WT1 over-expression is prevalent (102) and will be comparing the anti-tumor efficacy of transduced autologous Tcm cells or naive cells.

Rationale/safety of enhanced affinity receptors

A persistent challenge for adoptive T-cell therapy is that most naturally occurring T cells specific for unmutated self/tumor antigens persisting in the periphery have TCRs with a relatively low affinity for the target antigen (17). In some cases, even the highest affinity

antigen-specific TCR that can be identified from a large screen of multiple donors may have an insufficient affinity to mediate effective tumor cell lysis. This largely reflects negative selection of developing T cells in the thymus, which eliminates T cells that can recognize self-antigens with a high affinity (103). Thymic negative selection is essential to protect against autoimmunity; however, this process also eliminates T cells that might have the best chance of destroying malignant cells (17). Therefore, strategies to enhance the affinity of TCRs intended for use in TCR gene therapy for targeting tumor antigens have been developed. A large number of studies have demonstrated that enhancing TCR affinity can increase the overall antigen sensitivity (the 'functional avidity') of a T cell (104–109), and recent evidence suggests that the off-rate (Koff) in particular strongly correlates with *in vivo* T-cell activity (106). Furthermore, since the highest affinity MHC class I (MHC-I)-restricted TCRs can mediate antigen-specific T-cell activation in the absence of CD8, enhanced affinity TCRs can also be used to redirect the specificity of CD4 T cells to MHC-I-presented tumor antigens (109). CD4⁺ T cells have been shown to play an important role in tumor clearance, both through direct anti-tumor activity (110), and concurrently administered transduced functional CD4⁺ T cells recognizing the same target might provide a local cytokine milieu that can enhance the activity of transferred as well as endogenous tumor-reactive CD8⁺ T cells (109, 111, 112).

Although enhanced affinity TCRs have the potential to greatly increase the efficacy of adoptive T-cell therapy, modification of the antigen-specificity of a TCR comes with some risks. Therefore, successful implementation of this approach will rely on the development of reliable *in vitro* and *in vivo* models for assessing the safety of candidate receptors. One potential risk of TCR gene modification is the risk that the modified receptor will have acquired off-target cross-reactivity. Although in principle this risk exists for any non-autologous T-cell clone or TCR gene therapy construct since the TCR will not have undergone negative selection in the host, trials utilizing allogeneic HLA-matched donor T-cell clones post-HCT or unmodified TCR genes have not detected any clinical evidence of TCR cross-reactivity with other self-antigens (25, 53, 59). However, the risk of a TCR exhibiting cross-reactivity likely increases with enhanced affinity TCRs, as these TCRs have an altered reactivity with the antigen and have not been subjected to thymic selection. TCRs interact with pMHC through three complementarity determining regions (CDRs): CDR1 and CDR2 interact primarily with MHC and orient the TCR on the MHC molecule, while CDR3, which consists of the hypervariable junction formed during V(D)J recombination, interacts primarily with the presented peptide. The risk of cross-reactivity is likely to be highest for TCRs that contain affinity-enhancing mutations within CDR1 and CDR2, which would have a tendency to increase affinity by improving binding to the restricting MHC molecule and thus might still trigger the T cell, despite weaker interaction with an alternative peptide. This notion has been highlighted in a recent trial in which T cells, expressing a TCR that had been generated by *in vitro* mutagenesis using phage display and containing mutations in CDR2, were infused and mediated rapid and fatal toxicity from unpredicted cross-reactivity with a self-antigen expressed in the heart (113, 114). By contrast, enhanced affinity TCRs that contain mutations restricted to CDR3 should be less likely to exhibit cross-reactivity, since these TCRs retain the germline-encoded MHC contact residues of the parental TCR, which have been subjected to thymic selection.

With this in mind, we have been developing methods for generating enhanced affinity TCRs for gene therapy that limit affinity-enhancing mutations to CDR3. *In vitro* engineering with TCR chain mutagenesis throughout the variable domains of selected TCRs followed by yeast or phage display for selecting enhanced affinity mutations has been utilized to generate enhanced-affinity receptors with nanomolar (115) or even picomolar affinities (116). A mammalian (T cell) display system rather than yeast or phage has also been developed, as this allows evaluation of TCRs in a context in which they are signaling-competent and expressed in a native conformation in complex with CD3 subunits (117, 118). We have adapted this system for screening saturation mutagenesis libraries that contain every possible amino acid combination within a selected region of the CDR3 α or CDR3 β genes of tumor-specific TCRs. Enhanced affinity variants have been isolated from these libraries by cell sorting based on augmented intensity of staining with tetramer (117). Using such saturation mutagenesis of the CDR3 α region, we have now isolated enhanced affinity variants of our highest affinity human WT1-specific TCR (C4).

A second approach to generating enhanced affinity TCRs that we are developing involves transducing hematopoietic progenitor cells (HPCs) with the TCR α chain of a selected tumor antigen-specific TCR and then selecting *de novo* generated TR β chains that confer enhanced antigen-specific reactivity in an *in vitro* system (OP9-DL1 co-culture) for T-cell development that can recapitulate many aspects of thymic T-cell development. This approach is based on the following observations: (i) in TCR transgenic mice, an unusual $\gamma\delta$ -like CD4/CD8 double negative (DN) TCR $\alpha\beta^+$ population is selected *in vivo*, as well as *in vitro* on OP9-DL1 cells (119–121); (ii) these DN $\alpha\beta^+$ cells preferentially develop in TCR transgenic mice in which the cognate antigen is known to be expressed in the thymus, and *in vitro* if the cognate peptide epitope is present (120, 122); and (iii) this ‘lineage diversion’ to a DN TCR $\alpha\beta^+$ phenotype is the result of strong agonist signaling after β -chain rearrangement but prior to normal thymocyte β -selection, and occurs due to pairing of the rearranged β -chain with the enforced expression of a rearranged TCR α chain at this early stage of T-cell development when the TCR α locus is usually closed and the only TCR that can normally receive a strong agonist signal is a $\gamma\delta$ -T cell. We have found that expression of a tumor antigen-specific TCR α chain in HSCs followed by *in vitro* culture on OP9-DL1 cells in the presence of the cognate tumor antigen can result in lineage diversion to the DN TCR $\alpha\beta^+$ lineage for developing T cells expressing an endogenously rearranged TCR β chain that pairs with the transgenic TCR α and recognizes the cognate antigen with high affinity. Thus, a TCR β chain library can be generated from this DN TCR $\alpha\beta^+$ population, from which enhanced-affinity TCR β chains can be identified.

Enhanced affinity self/tumor-specific T cells can be safe *in vivo*

Enhanced affinity TCRs by definition have an affinity for self-antigen that would not normally be permitted by central tolerance mechanisms in the thymus. Bypassing the affinity thresholds set by thymic selection is not without risk, as it has the potential to increase the hazard of autoimmunity. For example, individuals that have mutations in the *Autoimmune Regulator* gene (*AIRE*), which is involved in expression of self-antigens for presentation in the thymus for negative selection, generally suffer from multi-organ autoimmunity (123). In the context of normal negative selection, lower affinity T cells

specific for self-antigens that do not cause autoimmunity can be readily isolated from healthy individuals (53, 124–126). Therefore, we were interested in determining if a window existed in which tumor antigen-specific TCRs that avoid negative selection and are found in the periphery of normal individuals can be safely affinity enhanced to increase the anti-tumor response without inducing autoimmunity. We addressed this question in a mouse model using TCRs specific for two different tumor antigens (WT1 and Mesothelin), which are both expressed at high levels in a large number of common human malignancies but expressed at only very low levels by few cell types in normal adult tissues (30, 31, 43).

We generated retroviral vectors encoding either wildtype affinity or enhanced affinity TCRs that we had generated and isolated specific for these self/tumor antigens, and found that enhanced affinity TCRs specific for these antigens can be expressed in T cells and safely transferred *in vivo* with retention of function and without induction of autoimmunity, even after *in vivo* stimulation in an inflammatory setting with a strain of *Listeria monocytogenes* encoding the target antigen (127). By contrast, following transfer of HSCs transduced with the enhanced affinity TCR genes into lethally irradiated hosts, transgene-expressing T cells that developed in the thymus showed signs of negative selection, with only cells that downregulated TCR expression and consequently functional avidity escaping deletion (127). Thus, while peripheral T cells transduced to express the most potent enhanced affinity TCR displayed enhanced functional avidity when compared to an intermediate affinity TCR or the parental TCR (Fig. 2A), retrogenic T cells (which were subjected to thymic selection) expressing the same high affinity TCR exhibited a functional avidity that was substantially lower than T cells expressing the lower affinity TCRs (Fig. 2B). These results likely reflect the high levels of expression in the thymus for some antigens such as WT1 that may model levels detected in embryonic rather than adult tissues, and suggest that it should be possible to enhance the affinity of tumor antigen-specific TCRs that can improve recognition of tumor targets but still not result in on-target autoimmunity in the normal tissues that express only low levels of the antigen. This phenomenon will almost certainly be dependent on the chosen target antigen, with a bias for proteins with limited normal tissue distribution and that are more important during fetal development than adult tissue functions.

Assessing the safety of enhanced affinity TCRs

Because of the risks associated with modifying the specificity of a TCR that will be used for TCR gene therapy, it is important to thoroughly assess the potential for cross-reactivity or on-target toxicities in normal tissues that express the target antigen before using such a reagent in patients. Cross-reactivity can be assessed by testing candidate TCRs for reactivity *in vitro* against cell lines generated from various tissue types that express the restricting MHC-I allele. However, since the most dangerous cross-reactive T cells reported in clinical trials have been reactive against rare tissue antigens (108, 113, 114, 128, 129), additional approaches will need to be developed to more precisely assess the potential for TCR cross-reactivity, such as *in silico* analysis of the human proteome for peptide sequences that preserve critical residues such as MHC-binding motifs and residues known to be essential for pMHC binding by the parental TCR (109).

To assess the potential for on-target WT1-specific toxicities, we have also utilized HLA-A2 transgenic mice (130). The mouse is particularly useful for studying WT1-specific TCRs, as WT1 expression and tissue distribution in mice is virtually identical to humans, the WT1_{126–134} peptide recognized by the A2-restricted C4 TCR is expressed in mouse as well as human WT1, and the epitope is known to be processed and presented by mouse cells (130, 131). To evaluate the safety of enhanced affinity human TCRs generated *in vitro*, the TCR chains are modified to contain mouse rather than human C α and C β domains for enhanced expression in murine T cells. Transduced T cells are then transferred into HLA-A2 transgenic animals, which can process and present endogenous WT1 peptides, and therefore would be susceptible to T-cell-mediated on-target off-tissue toxicity resulting from excessively high affinity targeting of WT1 by the transferred T cells.

Even this A2-transgenic *in vivo* screening system has limitations. It cannot assess cross-reactivity to the full range of human peptides that would be experienced in a patient, and indeed can never fully account for the fact that, even with unmodified TCRs advanced to therapy, the TCR will have been derived from a donor that has a unique set of selecting and deleting antigens distinct from other individuals not genetically identical. However, this A2-transgenic system has the benefit of allowing direct assessment of the risk of on-target off-tissue toxicity associated with increasing the sensitivity of the transduced T cells for a self-antigen, and it provides a large and diverse panel of HLA-A2 presented peptides for elucidating if an enhanced affinity TCR has an increased propensity for cross-reactivity.

Abrogation of negative regulators of T-lymphocyte function

Rendering prior to infusion tumor-reactive T cells resistant to inhibitory signals that may be encountered *in vivo* has the potential to increase T-cell survival, improve functional avidity, and sustain anti-tumor activity during T-cell therapy. Tumor-reactive T cells can readily be engineered to express dominant negative proteins or gene-specific siRNAs or targeted endonucleases (132) that ablate negative regulators of T-cell function, providing a tailored clinical approach that would be less toxic than administration of reagents that must globally target all host immune responses, such as the checkpoint blockade inhibitors anti-CTLA4 or anti-PDL1. The E3-ubiquitin ligase Casitas B-lineage lymphoma (Cbl-b) is a critical gatekeeper of T-cell activation that regulates both TCR and CD28 signaling in T cells (133, 134). We showed that abrogation of Cbl-b in murine tumor-reactive, *in vitro*-expanded, effector T cells (Teff) increased T-cell functional avidity, bypassed the requirement for CD28 signaling for IL-2 production by donor T cells, and overcame the requirement for administration of exogenous IL-2 to achieve tumor eradication following T-cell transfer (135). As observed in our clinical trials, efficacy in therapy correlates with T-cell persistence, and CD28 has been a surrogate marker for predicting T-cell persistence *in vivo* but is also often lost on human T cells that have been expanded *in vitro* and differentiated into Teff cells. Furthermore, even if infused T cells do retain expression of CD28, many tumors do not express the CD28 ligands CD80 and CD86, which results in inefficient T-cell activation by the tumor, making a strategy that provides a costimulatory signal without CD28 ligation particularly useful. Bypassing the requirement for exogenous IL-2 for tumor eradication also has distinct advantages, as IL-2 cannot be permanently administered, only transiently maintains T-cell persistence in patients (26, 136), and can also cause toxicity in

humans as well as induce inhibitory CD4⁺ Tregs (137). Thus, abrogating a single molecule, Cbl-b, can overcome multiple obstacles to effective T-cell therapy.

We have also begun pursuing abrogation of other inhibitory pathways that impact TCR signaling and antigen-specific tolerance. The phosphatase SHP-1 is rapidly recruited to the TCR complex after recognition of low affinity ligands (138), and enforces a negative feedback loop to prevent T-cell activation by dephosphorylation of multiple TCR signaling components. The induction of SHP-1 expression and recruitment to the TCR complex after recognition of self/tumor antigen may be a critical obstacle in tumor therapy in which antigen presentation can often be limiting. We showed in a mouse model of acute LCMV Armstrong infection that abrogating SHP-1 in naive virus-specific T cells increased the magnitude of short-lived Teff cells generated following acute viral infection without impacting memory formation (139). In further studies, we showed that abrogating SHP-1 in T cells increased the functional avidity of *in vitro*-expanded tumor-reactive Teff cells, enhanced the accumulation of short-lived Teff cells *in vivo* following transfer, and resulted in superior anti-tumor activity than wildtype T cells. Furthermore, despite the increase in short-lived effector T cells, the loss of SHP-1 did not impact the development of functional memory T cells (140). A recent study using human tumor-specific T cells showed that SHP-1 was upregulated in a TCR affinity-dependent manner, with the T cells expressing TCRs with supraphysiological affinity having the highest levels of SHP-1 (141). Thus, as we continue to develop tumor-reactive TCRs with higher affinity, it may be critical to also ablate SHP-1 to maximize *in vivo* effector function. Our preliminary studies have indicated that abrogation of another inhibitory signaling module, PD-1, in tumor-reactive Teff can also dramatically improve the expansion of short-lived effector T cells in a preclinical tumor therapy model, with resultant improvement in therapeutic efficacy (authors' unpublished observations).

Abrogating TGF β signaling in effector T cells prior to transfer is also a potentially useful approach as TGF- β is an immunosuppressive cytokine that is often highly expressed in solid tumors such as adenocarcinomas of the prostate (142) and pancreas (143). Effector T cells express high levels of the TGF- β receptor, can produce TGF- β (144, 145), and TGF- β signaling suppresses both the proliferation and cytolytic activity of T cells (146, 147). Recently, we showed that abrogation of TGF- β signaling in self/tumor-specific effector T cells increased the expansion, persistence, and infiltration of the T cells in an autochthonous prostate cancer mouse model engineered to express the model antigen ovalbumin in both malignant and normal prostate (148), overcoming substantial obstacles for effector T cells in therapy of this tumor. However, inhibition of TGF- β signaling in T cells was not sufficient to circumvent T-cell dysfunction due in part to chronic antigen signaling that could also not be rescued by PDL1 blockade. These results suggest that the extent of dysfunction of self/tumor-specific T cells during cancer therapy may be influenced by multiple mechanisms and that additional modalities will likely have to be employed to sustain activity of adoptively transferred T cells in solid tumor environments. Of note, although it is tempting to abrogate multiple inhibitory pathways concurrently, our preliminary results suggest that complete abrogation of multiple inhibitory proteins simultaneously may modify T-cell function in ways that can be counterproductive for T-cell function/survival, and testing will

be required to identify how much knockdown of each gene in concert is most effective. In short, these studies suggest that abrogating inhibitory pathways in tumor-reactive T cells can increase antitumor activity without causing toxicity or autoimmunity due to enhanced recognition of the low levels of self-antigen expressed on normal tissues, and should be explored in clinical trials in which T-cell function and/or persistence after transfer is not sustainable.

Broadening the use of adoptive T-cell immunotherapy to additional solid tumors

Pancreatic ductal adenocarcinoma (PDA), the predominant neoplasm of the pancreas, is the fourth leading cause of cancer-related mortality in the United States (149). The vast majority of patients (~85–90%) present with tumors not amenable to surgical resection (150), resulting in a median survival after diagnosis of <1 year (151). Even in patients that undergo resection with curative intent, the overall 5 year survival rate is still dismal (~20%) due to local recurrence and metastasis (152–155). The lethality of this disease is attributed to the advanced stage of disease at diagnosis, a propensity for metastasis, and a marked resistance to systemic therapies, as most therapies for PDA have until very recently either failed in clinical trials or imparted only modest survival benefit (156).

Tailoring adoptive T-cell therapy for targeting PDA *in situ* could hopefully mimic the success achieved in malignant melanoma (26, 38). However, the pancreatic tumor environment poses unique obstacles to sustaining effector T-cell activity. PDA produces a highly compact and sclerotic mass. This robust fibroinflammatory stromal reaction is comprised of fibroblasts, myofibroblasts, pancreatic stellate cells, immune cells, and endothelial cells all embedded in a dense extracellular matrix (ECM) that contains immunomodulating growth factors and cytokines (156, 157). Surprisingly, the non-malignant stroma comprises the majority of the tumor mass, while neoplastic epithelial cells are only a minor constituent of tumors (~10%) (157). The desmoplastic stroma, including the abundant ECM, has recently been implicated as a primary mechanism of drug resistance (108, 158, 159), and therefore, we predict it will also greatly impact the functional activity of PDA-reactive T cells due to its paradoxically inflammatory and immunosuppressive nature.

One potential barrier to immunotherapy of PDA that attempts to harness the endogenous immune response against PDA may be a lack of immunogenicity. In contrast to melanoma (160) and lung cancer (161), which contain ~200 nonsynonymous mutations per tumor (162), PDA has relatively few coding mutations, estimated at ~30–50 per tumor (162, 163). The checkpoint blockade inhibitors anti-CTLA-4 or anti-PD1 have induced clinical responses in melanoma, and anti-PD-1 has induced responses in lung cancer (164, 165), suggesting that the high frequency of coding mutations in these malignancies may reveal unique foreign epitopes that can be effectively targeted by endogenous T cells. Similar attempts at modulating endogenous immunity in PDA have been minimally effective (166–168), supporting the view that PDA may be much less immunogenic. However, despite the apparent lack of immunogenicity and the unique characteristics of the tumor stroma, vaccination of PDA patients with an irradiated allogeneic pancreatic tumor cell vaccine

engineered to express GM-CSF (GVAX) induced PDA-reactive T-cell responses in a fraction of patients that were associated with improved outcomes (169–171). Analysis of patients' peripheral T cells revealed specific responses to an overexpressed self-antigen, Mesothelin (MSLN) in the fraction of vaccinated patients that had improved outcomes. Since not all patients mounted detectable endogenous MSLN-specific T-cell responses post-vaccination, infusion of *ex vivo* generated MSLN-specific T cells could help clarify if more potent T-cell responses to this antigen would be associated with better clinical outcomes.

MSLN is overexpressed in virtually all PDAs but not in normal pancreas (46), is immunogenic in humans (170, 172, 173), and is believed to contribute to the malignant phenotype by promoting tumor migration and invasion (44, 45). Although MSLN is expressed at low levels in mesothelial cells of the pleura, peritoneum and pericardium (43), MSLN^{-/-} mice develop normally and have no phenotype, indicating it is not essential for life (174). Since MSLN contributes to oncogenicity, loss of expression is predicted to be disadvantageous for the tumor, reducing the potential for tumor escape. Therefore, we are currently isolating MSLN-reactive T-cell clones and TCRs from HLA-A1⁺ and HLA-A2⁺ donors to identify TCRs that could be used in planned clinical trials targeting MSLN. However, as targeting a single antigen may allow for escape, we plan to also examine targeting of WT1, which is over-expressed in ~75% PDAs but not normal pancreatic cells (175) and for which we already have a high affinity human WT1-specific TCR. Intriguingly, treatment of human PDA with gemcitabine, the standard of care for PDA, increases antigen presentation, sensitizing the tumor cells to lysis by WT1-specific CTLs (176). This observation suggests that combining T-cell therapy with chemotherapy has the potential to improve activity of donor T cells.

There has been a vast disconnect between the success of therapies obtained from *in vitro* assays and transplantable tumor mouse models (xenograft or orthotopic) and the failure of such therapies in PDA patients (177). Not surprisingly, the immune response also differs dramatically in response to transplantable tumors as opposed to a tumor that arises within a normal tissue from a preinvasive state (2). To reconcile the discrepancy between clinical results and transplantable models, and create preclinical models that will be instructive for designing human trials, genetically engineered mouse models (GEMM) of PDA were designed, in which the identical driver mutations found in the majority of human PDAs were expressed at physiological levels in the murine pancreas. Expression of an activating point mutation in one Kras allele (*Kras*^{G12D}) in pancreatic progenitor cells was sufficient to induce the full range of preinvasive intraepithelial neoplasms (PanINs) that ultimately culminate in invasive and metastatic PDA (178). Addition of a loss of function point mutation in one Trp53 allele (*p53*^{R172H}) in combination with the *Kras*^{G12D} mutation increased the rate of malignant progression in *Kras*^{G12D/+}; *Trp53*^{R172H/+}; *Pdx-1*Cre (*KPC*) mice and the propensity for metastasis (179). This model faithfully recapitulates the cardinal features of the human disease including the natural history, genetic progression, histopathology, and drug resistance and now serves as a primary platform for rational therapy design for PDA (108, 158, 159, 178–180).

The tumors in *KPC* mice, similar to human PDA, have extensive desmoplasia (Fig. 3A) and infiltration with immunosuppressive cells, presenting substantial potential obstacles to the

efficacy of engineered tumor-reactive T cells. PDA contains a large fraction (>50%) of CD45⁺ cells of hematopoietic origin that include diverse subsets of immunosuppressive myeloid cell populations (181, authors' manuscript submitted) (Fig. 3B) and CD4⁺FoxP3⁺ regulatory T cells that could also inhibit activity of adoptively transferred T cells (181) (Fig. 3C). We have found that primary tumor epithelial cells derived from *KPC* mice uniformly express high levels of MSLN and a large fraction of the cells also express high levels of WT1 (authors' unpublished observations), making it feasible to target these naturally overexpressed candidate human tumor antigens rather than model antigens, which should more adequately emulate viable clinical strategies.

We have generated murine MSLN-specific T cells, isolated the TCRs, and inserted these into retroviral vectors for pursuing preclinical studies in the autochthonous *KPC* model. The MSLN-specific T cells were generated by immunizing mice with an attenuated adenovirus vaccine vector engineered to express recombinant murine MSLN (Ad-MSLN). Although only a low frequency of MSLN-specific T cells was induced in mice by Ad-MSLN (approximately 2.5-fold higher than control mice) (Fig. 3D), after expansion *in vitro* most CD8⁺ T cells bound tetramer (Fig. 3D). As discussed earlier, one obstacle to targeting overexpressed self/tumor antigens is that TCRs isolated from the normal repertoire can have suboptimal affinity due to deletion of cells during development. Therefore, our laboratory has developed technologies to increase the affinity of TCRs isolated from endogenous tumor-reactive T cells. To model the impact of an enhanced affinity TCR reactive to MSLN, we also immunized MSLN^{-/-} mice, anticipating that the lack of central tolerance in this setting would allow us to isolate higher affinity TCRs. Indeed, all of the MSLN-reactive T-cell clones isolated from MSLN^{-/-} mice expressed similar levels of TCR chains as T cells isolated from wildtype mice but exhibited increased tetramer staining consistent with expressing higher affinity TCRs (Fig. 3E). We have isolated these TCRs, generated retroviral vectors, and engineered murine CD8⁺ T cells to express the introduced TCRs. We are currently pursuing studies in *KPC* mice with defined primary tumor burden (2–5 mm diameter as determined by high-resolution ultrasound) to determine the requirements for achieving clinically beneficial antitumor-activity (authors, manuscript in preparation) (Fig. 3F). These studies will provide a framework for our design of upcoming clinical trials in human PDA.

Bench back to bedside

Based on strategies designed and tested in preclinical mouse models, approaches for overcoming some of the obstacles to efficacy with adoptive T-cell therapy have been identified and validated, including methods to improve T-cell persistence *in vivo* after transfer and to increase the avidity of the infused T cells by enhancing the affinity of self/tumor-reactive TCRs introduced into the T cells for use in therapy. Providing IL-21 *ex vivo* during the early expansion phase of the tumor-reactive T cells, expressing the tumor-reactive TCRs in CD8⁺ T cells with central memory characteristics, and/or increasing TCR affinity, may provide more predictably potent and persistent responses for the treatment of leukemia (pre- or post-HCT). The safety and potential efficacy observed in our clinical studies support expanding efforts with adoptive T-cell therapy to treat other malignancies, particularly employing strategies described here for establishing persistent responses to validated targets

such as WT1. Preclinical studies are now underway in our lab to adapt this technology to treat solid tumors such as PDA and lung cancer, and suggest that additional modifications to T cells such as abrogation of inhibitory pathways may prove beneficial. Understanding and overcoming the obstacles for effectively implementing T-cell therapy for solid tumors can greatly benefit from the use of genetically engineered mouse models that faithfully recapitulate the hallmark features, including tumor antigenicity, of the human disease. Studies in such models provide the unique opportunity to assess the impact of changing individual parameters, and should facilitate design of strategies for testing in clinical trials that have an increased chance of achieving efficacy. Checkpoint blockade inhibitors and tumor vaccines are showing increased evidence of therapeutic activity in cancer patients, and learning how to best use such reagents and strategies in concert with adoptive T-cell therapy has the potential to greatly enhance therapeutic outcomes. Our efforts to continue to use relevant preclinical models of human disease to design and test therapeutic strategies, combined with detailed immunologic monitoring in patients entered on clinical trials, should make it possible to achieve more effective T-cell therapy regimens and expand this approach for a broader scope of malignancies.

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Fig. 1. Lessons learned from the clinic

(A) Mean effective concentrations of peptide required to achieve 50% lysis of TAP-deficient HLA A*0201⁺ B-lymphoblastoid cell lines (T2 B-LCL) pulsed with titrating doses of peptide at an effector to target ratio (E:T) of 10:1 by the CD8⁺ T-cell clones infused into each patient. Arrows indicate the avidity of the clones infused into patients described in Fig. 1B (1) and Fig. 1C (2), respectively. (B) Percent of leukemic blasts in the blood (grey bars) measured at indicated timepoints before and after the infusion of 3.3×10^9 cells/m² WT1-specific CD8⁺ T cells (vertical arrow). (C) Percent multimer⁺CD8⁺ T cells (y-axis) among

total CD8⁺ T cells (solid circles) and in BM (open circles) collected before and after infusions. Vertical arrows indicate time of infusions.

Fig. 2. Peripheral T cells transduced with enhanced-affinity TCRs surpass the threshold of thymic selection

(A) Splenocytes from P14 mice were transduced to express the wildtype-affinity WT1-specific TCR from clone 3D (3D-*wt*), which was the highest affinity clone that could be isolated from the normal peripheral T cell pool, or one of two CD8-independent enhanced affinity variants of 3D-*wt*: 3D-NYH (intermediate affinity), and 3D-PYY (highest affinity). (B) Splenocytes were isolated from retrogenic mice generated by transducing RAG2^{-/-} bone marrow HSC with the 3D-*wt* or enhanced-affinity TCR constructs, followed by transfer into lethally irradiated B6 recipients. TCR-transduced P14 T cells (A) or retrogenic T cells (B) were analyzed for relative TCR transgene expression as measured by Vβ10 staining, and for staining with WT1 tetramer. Transduced P14 T cells or retrogenic T cells were also stimulated with WT1 peptide, expanded for 1 week *in vitro*, and then assayed for functional avidity by measuring interferon-γ production in response to antigen-presenting cells pulsed with decreasing concentrations of WT1 peptide.

Fig. 3. Preclinical modeling to maximize therapeutic efficacy of T cells during adoptive immunotherapy of PDA

(A) Histopathology of PDA in representative sections from *KPC* mice demonstrates extensive desmoplasia as indicated by Masson's trichrome staining of collagen in the ECM. (B) Influx of distinct subsets of CD11b⁺ myeloid cells to the spleen and tumors of *KPC* mice compared to non-tumor bearing mice. Plot is gated on CD45⁺ cells. (C) CD4⁺FoxP3⁺ Tregs accumulate in PDA in *KPC* mice. CD45⁺CD4⁺ cells isolated from collagenase-digested *KPC* tumors were analyzed for expression of CD25 and FoxP3. (D) Frequency of

tetramer-binding MSLN-specific CD8⁺ T cells in wildtype mice before and after immunization with Ad-MSLN, and following two cycles of *in vitro expansion* of MSLN-reactive splenocytes from immunized mice stimulated with MSLN and IL-2. (E) A comparison of the mean fluorescence intensity (MFI) of TCR chain (V β) staining and tetramer staining of MSLN-specific T-cell clones isolated from T cell lines derived from immunized wild type (black bars) and MSLN^{-/-} (red bars) mice. (F) Representative high-resolution ultrasound (Vevo 2100) of a pancreatic mass in the body of the pancreas of a 4 month old *KPC* mouse that met the enrollment criteria for T-cell therapy. PV, portal vein; T, tumor.