Expert Opinion

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Adoptive T-cell therapy for the treatment of solid tumours

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Solid tumours can be eradicated by infusion of large amounts of tumour-specific T-cells in animal models. The successes seen in preclinical models, however, have not been adequately translated to human disease due, in part, to the inability to expand tumour antigen-specific T-cells *ex vivo*. Polyclonality and retention of antigen-specificity are two important properties of infused T-cells that are necessary for successful eradication of tumours. Investigators are beginning to evaluate the impact of attempting to reconstitute full T-cell immunity representing both major T-cell subsets, cytolytic T-cells and T-helper (Th) cells. One of the more important and often overlooked steps of successful adoptive T-cell therapy is the *ex vivo* expansion conditions, which can dramatically alter the phenotype of the T-cell. A number of cytokines and other soluble activation factors that have been characterised over the last decade are now available to supplement *in vitro* antigen presentation and IL-2. Newer molecular techniques have been developed and are aimed at genetically altering the characteristics of T-cells including their antigen-specificity and growth *in vivo*. In addition, advanced imaging techniques, such as positron emission tomography (PET), are being implemented in order to better define the *in vivo* function of *ex vivo* expanded tumour-specific T-cells.

Keywords: cytotoxic T-cells (CTL), cancer, cytokine, dendritic cells, *ex vivo* expansion, T-helper cell, polyclonal, T-cell receptor, vaccine

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1. Introduction

Animal models demonstrate that adoptive T-cell therapy of advanced stage malignant disease is a feasible and successful treatment strategy. Increased understanding of the complex nature of the immune effector cells and the identification of tumour antigens is providing researchers with the appropriate tools to generate and reconstitute effective tumour-specific immunity through adoptive transfer of T-cells. *Ex vivo* expansion has been problematic and many hurdles will need to be overcome. Two advances in T-cell culture have improved the ability to generate tumour-specific T-cells *ex vivo*. The first was to increase antigen-primed T-cells *in vivo* prior to *ex vivo* expansion by active immunisation. The second was the improvement of culture conditions with the use of recently identified tumour antigens, cytokines and co-stimulatory molecules in conjunction with IL-2. In addition, technologies are being developed to genetically modify T-cells to create the appropriate immune microenvironment for tumour destruction. A better understanding of immunoregulatory mechanisms will allow us to overcome tumour-induced immunosuppression in cancer patients during adoptive T-cell therapy.

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2. The clinical role of adoptive T-cell therapy

Adoptive T-cell therapy is an immune-based therapeutic strategy that will significantly boost tumour-specific T-cell immunity above that observed by vaccination alone [1]. Transferred T-cells can potentially represent a major fraction (1:2) of the host's lymphocytes [1]. This strategy could have an advantage over active vaccination for the treatment of more extensive malignancy where greater control of the numbers of tumour-specific T-cells may be required. Adoptive T-cell therapy techniques that have shown great promise in human clinical trials have been applied to the Epstein-Barr virus (EBV)-related disorders, immunoblastic lymphoma and Hodgkin's disease [2-4]. These studies, as will be further discussed, provide proof of principle that administration of *ex vivo* expanded T-cells can reconstitute effective long lasting immunity *in vivo*. However, the major distinction between the treatment of EBV-malignancies and non-viral malignancies is the targeting of non-self viral antigens. In the most common solid tumours, self proteins are the target antigens of T-cells. Thus, *ex vivo* expansion of these T-cells is further complicated by the probability that the most potent and robust self tumour antigen-specific T-cells recognising self antigens have either been deleted or rendered ineffective by tolerising mechanisms [5,6]. *Ex vivo* expansion methods must be optimised to maximise functional effects of the remaining self-reactive T-cells while preventing the outgrowth of non-specific immune effector cells. Recent evidence demonstrating that single clones are ineffective at mediating tumour eradication further suggests that *ex vivo* expansion methods should also be designed to maintain polyclonality in order to ensure multiple specificities for the same epitope as well as multiple T-cells subsets specific for the target antigen [7]. The need for polyclonal responses is particularly important for a number of reasons, including the prevention of antigen-loss variants, the prevention of major histocompatibility complex (MHC) class I loss variants and the need of helper activity by cytotoxic T-cells (CTL) for expansion and persistence *in vivo*. Much of our current thinking of the obstacles of adoptive T-cell therapy stems from mouse models and the results of previously reported human clinical trials.

3. Human clinical trials of adoptive T-cell therapy

Clinical trials of adoptive T-cell therapy arose from promising earlier studies evaluating the antitumour efficacy of lymphokine activated killers (LAK). LAK are generated from patient peripheral blood mononuclear cells (PBMC) with IL-2 and are capable of killing tumour cells in a non-MHC-restricted fashion. Clinical trials, utilising LAK, have been carried out by several groups treating a variety of carcinomas, including melanoma, ovarian, renal cell and colorectal [8-10]. The low response rates ranging from 0 - 20% following infusion of LAK are likely a reflection of the inability of the LAK to home to tumour sites and the lack of specificity for the tumour. However, the response rates were encouraging and provided impetus for identifying lymphocyte populations, mainly T-cells, that have increased specificity and antitumour effector function.

The strategies which have been developed for increasing the number of tumour-specific T-cells, *ex vivo*, have included the non-specific expansion of tumour infiltrating lymphocytes (TIL), the non-specific expansion of sensitised lymph nodes draining the tumour site and the expansion of tumour antigen-specific T-cell populations. The first two strategies are based on the hypothesis that there is an increased precursor frequency of tumour-specific T-cells at sites local to the tumour or in the lymph node draining the tumour. The last strategy exploits the use of known tumour antigens to expand specific T-cell populations.

Beginning in the late 1980s, clinical trials were carried out using *ex vivo* expanded TIL from a variety of cancers including malignant melanoma, renal cell carcinoma, breast cancer and lung cancer. Response rates after TIL therapy were variable and ranged between 0 - 60% with most being between 10 - 25% [11]. Studies using TIL have been difficult to perform in cancer patients due to:

- The limitations in obtaining significant amounts of tumour from which to derive the cells.
- The inability to expand autologous cancer target cell lines to test T-cell lytic activity prior to infusion.
- The location of metastatic relapse (e.g., bone, lung, brain and liver) prevents tumour cell and thus T-cell harvest.

TIL are typically expanded by incubating the cells with very high concentrations, up to 7000 U/ml, of IL-2. These culture conditions typically result in enriching for T-lymphocytes with LAK-like activity. For example, Belldegrun and colleagues reported the results of a study characterising the cell populations derived from *ex vivo* expansion of lymphocytes infiltrating human renal cell cancer [12]. Renal TIL expanded in high doses of IL-2 are predominantly CD3+ but display lytic activity similar to LAK, including high activity against K562, Daudi and allogeneic tumour cells. Ratto and colleagues observed similar findings following expansion of lung TIL [13].

The use of IL-2-expanded TIL in melanoma trials has led to observations that could have important implications for the design of future adoptive T-cell trials. Rosenberg and colleagues reported the results of a clinical trial evaluating treatment of melanoma patients with both TIL and high dose bolus IL-2 [14,15]. Of the 86 patients treated, 24 partial and five complete responses were observed. A number of important correlations were observed when comparing the *in vitro* characteristics of the expanded TIL of responders with the TIL of the non-responders. Clinical responses were associated *in vitro* cellular responses such as higher specific lysis of autologous tumour targets, shorter doubling times, younger cul-

tures and increased autologous tumour-specific granulocyte-macrophage colony stimulating factor (GM-CSF) secretion [15].

Like TIL, lymph nodes draining either a tumour or vaccine site represent another potentially rich source of tumour-specific T-cells. Clinical response rates following reinfusion of *ex vivo* activated nodal T-cells are similar to TIL infusions [11]. In a recent Phase I study, To and colleagues evaluated the toxicity and clinical responses of infusions of *ex vivo* expanded vaccine-draining lymph node-derived lymphocytes in patients with head and neck cancers [16]. Fifteen patients were vaccinated on the thigh with irradiated autologous tumour with GM-CSF. After 8 - 10 days, the inguinal, vaccine-draining lymph nodes were harvested and activated *ex vivo* with staphylococcal enterotoxin A (SEA) and expanded in high dose IL-2. The resulting cells were mainly CD3+ and had mixed CD8+ and CD4+ phenotype. Toxicity following reinfusion was minimal and limited to grade 2. Of the 15 patients, only two responded with one patient being disease free. In a similar study, patients with newly diagnosed gliomas were immunised with autologous tumour cells followed by harvesting and expansion of the vaccine-draining lymph nodes [17]. The trial resulted in four partial responses out of 12 patients treated. Further *in vitro* characterisation of the resulting T-cell populations can provide more insight into how this promising technique can be optimised. As an example, perhaps increased tumour killing could be achieved by including specific tumour antigens during expansion.

Recent advances in molecular and cellular immunology provide the technology needed to identify and define tumour-specific antigens, as well as an understanding of how T-cells recognise antigens. It is presumed that a highly purified, antigen-specific and polyclonal T-cell population would be the most efficient strategy for tumour eradication. Clinical trials testing the feasibility of antigen-specific T-cell therapy for the treatment of disorders and cancers associated with EBV have been reported. EBV-specific CTL lines have been generated *in vitro* from donor blood and used successfully to treat B-cell lymphoproliferative disorder in bone marrow transplant recipients [4]. In addition, EBV-specific CTL have been expanded *ex vivo* and reinfused into patients with advanced stage relapsed Hodgkin's disease [2]. This feasibility study demonstrated:

- CTL could be expanded from patients with advanced cancer.
- T-cells were found to persist up to 13 weeks after infusion.
- Antigen-specific T-cells were detected in the pleura at levels 10-fold higher than the peripheral blood, implying trafficking of infused T-cells to sites of tumours.
- Transferred T-cells produced increased EBV-specific cytotoxic activity in peripheral blood lymphocytes as measured by chromium release assay and a decrease in peripheral blood viral burden as measured by quantitative PCR.

A preliminary report of the clinical status of patients receiving this treatment indicated that in addition to reducing viral burden, the T-cell infusion may stabilise or reduce disease symptoms [18] suggesting that Phase II clinical trials should be carried out to evaluate clinical responses.

Recently, Dudley and colleagues assessed the safety, feasibility and clinical response of adoptive T-cell therapy of melanoma using *ex vivo* expanded CD8+ cytolytic T-cell clones specific for an HLA-A2 binding peptide derived from the melanoma antigen GP-100 [7]. Twelve patients were treated with multiple infusions of GP-100-specific T-cell clones with an average of 1 x 10^{10} cells/infusion. The T-cells clones were selected based on their apparent avidity for peptide antigen as assessed by the magnitude of antigen-specific cytokine release. Although the clones secreted large amounts of IFN-γ and recognised HLA-A2+ melanoma cell lines, only two patients had minor partial responses. An important finding from this study was that the T-cells disappeared rapidly and were undetectable at 2 weeks, even though the patients received concomitant iv. IL-2. The authors postulated several mechanisms to explain the lack of clinical effects. One possibility is that the CD8+ T-cell clones could not persist in the absence of sufficient help, and the authors suggested that the treatment could be improved by co-infusion with antigen-specific CD4+ T-cells. This is supported by previous studies by Walter and colleagues who observed that CMV-specific CTL clones declined more rapidly in patients deficient in CD4+ Th cells specific for CMV [19].

These clinical trials as well as many others identify major areas that should be further investigated to improve the clinical outcome of adoptive immunotherapy. Namely, identification of *ex vivo* expansion conditions and other novel molecular techniques aimed at improving the antigen-specificity, functionality, polyclonality and longevity of the infused T-cells.

4. *Ex vivo* expansion of tumour-specificT-cells

The success in expanding EBV-specific T-cells from peripheral blood of patients may be related to the endogenous, naturally occurring high precursor frequency of these cells, thus, the abundance of viral-specific T-cells in the initial cultures. In contrast to viral cancers, T-cells directed against non-viral tumours are much less abundant [20]. *Ex vivo* expansion of tumour antigen-specific T-cells may be greatly facilitated by prior immunisation against specific tumour antigens. The authors recently demonstrated the feasibility of this approach in breast and ovarian cancer patients who were vaccinated against HER-2/neu with a helper peptide-based vaccine [21]. Nineteen patients were vaccinated with a HER-2/neu peptide-based vaccine consisting of three helper peptides, each of which contained an HLA-A2 binding motif, fully nested within its sequence. Six monthly vaccinations with GM-CSF as adjuvant resulted in increased levels of T-cells specific for the encompassed HLA-A2 motifs to levels similar to those measured against influenza and CMV HLA-A2-binding peptides. The increased precursor frequency after vaccination improved the generation of T-cell clones specific for the HER-2/neu HLA-A2 binding peptide, p369-377. As an

example, in one patient, a total of 21 p369-377 clones were generated. With the exception of two clones, all clones were CD3+ [22]. Eleven of the clones were CD8+/CD4–. Nine of the clones were CD4+/CD8-, despite being specific for an HLA-A2 binding peptide. The remaining five clones contained varying levels of both CD4+ and CD8+. The majority (19/21) of clones expressed the α/β T-cell receptor but interestingly, two clones expressed the γ/δ T-cell receptor [23]. Several of these clones could be induced to secrete IFN-γ in response to p369-377 peptide stimulation. Several clones were able to lyse HLA-A2-transfected HER-2/neu-overexpressing tumour cells, including the γ/δ TCR expressing clones. Similarly, Reddish and colleagues demonstrated that breast cancer patients can generate MHC class I-restricted CTL against MUC-1-expressing adenocarcinomas following vaccination with a MUC-1 helper peptide [24]. Investigations such as these demonstrate that *ex vivo* expansion and characterisation of cancer specific T-cells is facilitated by vaccination and that the responses elicited to vaccine can be diverse and polyclonal.

Several techniques for *ex vivo* expansion of tumour-antigen T-cells are currently being developed in various laboratories. Two variables that can be manipulated during *ex vivo* expansion are the antigen and cytokine environments. The identification of tumour-specific antigens, as well as the important tumour-responding T-cell populations, has ushered in a new era of cellular expansion techniques that can generate T-cell lines and clones with increased antigen-specificity. Manipulating the cytokine environment also allows for the preferential expansion of T-cell subsets such as CD4+ and CD8+ or Th1 and Th2. Antigen-specific techniques are preferable over non-antigen-specific techniques due to the fact that even with vaccination, antigen-specific T-cell precursors may not be at sufficient levels to expand preferentially during stimulation with non-specific activation such as with anti-CD3/anti-CD28 beads or bacterial products such as SEA. This is evident from previous studies demonstrating that expansion of tumour-infiltrating T-cells with non-specific methods does not promote expansion of tumour-specific T-cells that are therapeutic *in vivo* [16,17]. These methods may activate all T-cells to a similar degree and may result in the expansion of non-specific bystander T-cells, immunosuppressive T-cells, or tolerated, non-functional T-cells. Methods are now being established allowing for selective expansion of specific T-cell subsets.

Adoptive T-cell therapy strategies have largely focused on the *ex vivo* generation of CTL due to observations that most tumours express MHC class I but not MHC class II and that CTL can mediate tumour regression in mice. Studies demonstrating the weak persistence of transferred CTL have led to investigations on how to extend their lifespans. Recent evidence from our laboratory suggests that simultaneous generation of tumour antigen-specific CD4+ Th cells could prolong the life of CTL *in vivo* [21]. The authors observed that > 60% of patients immunised with HER-2/neu helper epitopes, each containing an encompassed HLA-A2 epitope, were able to develop HER-2/neu specific CD8+ T-cell immunity. The CD8+ T-cell response was maintained, in some patients, for at least one year following vaccination. In contrast, 2/5 (40%) patients immunised with a single HER-2/neu HLA-A2 9-mer peptide, p369-377 (E75) developed HER-2/neu CD8 T-cell immunity that declined to undetectable levels within 5 months of the last vaccination (Knutson & Disis, unpublished observations). These data are consistent with findings in murine viral models where persistence of CD8+ T-cells is critically dependent on concurrent CD4+ T-cell immunity [25]. For example, in the murine LCMV model of viral immunity, loss of CD4 T-cell help results in impaired memory phase CTL responses leading to the inability of the mice to permanently control infection [26-28]. Moreover, CD4+ T-cells may also possess direct and indirect killing properties [29]. CD4+ T-cells encountering tumour directly or indirectly through dendritic cell (DC) cross-presentation can release a wide variety of cytokines, such as TNF-related apoptosis-inducing ligand (TRAIL), which can activate apoptotic pathways in tumour cells [30,31].

The *ex vivo* expansion of tumour antigen-specific CD4+ T-cells has been impeded by the lack of defined MHC class II-restricted tumour antigen peptides and the appropriate cytokine environment optimal for the generation of CD4+ T-cells capable of eliciting an inflammatory or Th1 type response. The authors had previously identified several MHC class II peptides, derived from HER-2/neu [32]. It was determined which of these peptides were relevant immunogens based on their ability to induce T-cells that recognised naturally processed HER-2/neu protein antigens in breast, ovarian and lung cancer patients [33]. Thus, with the identification of relevant MHC class II antigens, an important objective was to define the appropriate cytokine environment that preferentially promotes the expansion of HER-2/neu-specific CD4+ T-cells with a Th1 type profile, a phenotype important to the development of a cell-mediated immune response at the tumour site. Th1 cells could be efficiently cultured by the inclusion of IL-12 along with peptide and IL-2 during culture. IL-12 is a heterodimeric cytokine, produced by B-cells, macrophages and professional antigen presenting cells (APC), that has multiple effects on CD8 T-cell function when added together with low-dose IL-2 [34-39]. As a model MHC class II antigen, the authors chose p776-790 derived from the intracellular domain of HER-2/neu. The majority of patients immunised with this peptide developed immunity to HER-2/neu protein. Furthermore, this epitope was commonly associated with epitope spreading suggesting natural presentation [40]. While immunity to p776-790 could be readily measured in short-term cultures, cell lines generated by *in vitro* stimulation with peptide and IL-2 as the only added cytokine resulted in no antigen-specific expansion. The inclusion of IL-12, along with IL-2, restored antigen-specific responsiveness in a dose-dependent fashion [41]. The resulting p776-790-specific T-cells responded readily to antigen by proliferating and producing Type I cytokines (IFN-γ and TNF-α). The

increased proliferative response of the cultures was due in part to an increase in the number of HER-2/neu-specific T-cells as assessed directly by ELIspot analysis. Inclusion of IL-12 into the cultures also resulted in a significant decrease of non-specific cellular proliferation. These results suggest that IL-12 is an important cytokine for *ex vivo* recovery and maintenance of antigen-specific CD4+ T-lymphocytes that would otherwise be lost by using IL-2 as the only cytokine.

IL-7 has shown promise for the expansion of CTL under certain *ex vivo* conditions. IL-7 is a stromal cell-derived cytokine and is associated with the early development of lymphoid cells. IL-7 activates the proliferation of naive T-cells and has been implicated as a key cytokine in maintaining homeostatic proliferation *in vivo* [42]. Recent findings suggest that the addition of IL-7 to cultures can promote preferential expansion of antigen-specific T-cells [43]. TIL derived from follicular lymphoma (FL) typically lack tumour-specific activity which is not recovered by culturing cells with FL along with IL-2. However, when TIL are preactivated through CD40 followed by exposure to FL they can be further expanded by inclusion of IL-7 along with IL-2. The expanded T-cells have greatly enhanced FL-specific CTL activity. The effects of IL-7 appear however to depend on the *ex vivo* expansion environment. In the lab the authors have found that when IL-7 is included along with an influenza matrix peptide and IL-2, peptide-specific lysis is reduced by 25 - 30% compared to cells cultured with peptide and IL-2 alone (Knutson, unpublished observations). The background, non-specific lysis was also increased 3-fold. This lack of effect of induction of peptide-specific responses may not be translatable to other peptide systems. For example, Tsai and colleagues have found that IL-7 potentiated the ability of peptide-pulsed DC to generate CTL responses against viral and tumour epitopes [44]. These discrepancies in outcome clearly point to the need to optimise the use of cytokines in preclinical studies prior to clinical trials.

Recent studies with IL-15 demonstrate that this cytokine can have important effects on *ex vivo* expansion of peptide- and protein-specific T-cells. IL-15 is structurally similar to IL-2 and their receptors share the IL-2Rβ and IL-2γ chains [45]. The IL-2Rα (CD25) and IL-15Rα chains confer specificity. Like IL-2, IL-15 is a pleiotropic cytokine and induces proliferation and functional changes of multiple haematopoietic cells including αβT-cells, γδT-cells, DC and NK cells [46]. IL-2 predisposes T-cells to undergo activation-induced cell death (AICD) and IL-15 promotes the generation of memory CD8+ T-cells [47]. The death-inducing effects of IL-2 are particularly important for the expansion of CD4+ T-cells which are extremely sensitive to IL-2 following antigen stimulation. IL-2 and IL-15 also can change the homing capabilities of cultured CD8+ T-cells [48]. Antigen-primed murine CD8+ T-cells cultured in IL-15 but not IL-2, preferentially home to lymphoid tissue such as spleen and lymph nodes, while IL-2 cultured CD8+ T-cells home to sites of inflammation but not lymphoid tissue. IL-15 cultured cells home to sites of inflammation to a lesser extent but mediate a robust antigen recall response. Currently it is unknown if these findings can be extrapolated to human T-cells but could have important implications in designing expansion conditions to generate T-cells capable of targeting lymph node disease. Pharmacological generation of an inflammatory response with anti-apoptotic agents may be considered in order to attract IL-2-cultured cells to tumour site.

Activation of T-cells directly through CD40 or indirectly using trimeric CD40L also enhances the expansion of antigen-specific T-cells [49]. CD40L is expressed on T-cells and is the ligand for CD40 which is expressed on antigen-presenting cells, including DC [50]. The interaction of CD40 with CD40L is IL-2- and CD28- independent and results in increased proliferation of T-cells as well as increased expression of Th1 cytokines (IFN-γ, TNF-α) [49]. During induction of tumour-specific immunity, CD40 activation promotes long-term survival of tumour-specific CD8+ T-cells [51]. Terheyden and colleagues have recently reported encouraging results demonstrating the utility of CD40 ligation during *ex vivo* expansion of melanoma-specific CD8+ T-cells and CD4 T+ cells derived from TIL [52]. When T-cells were cloned from TIL using oncolysate-pulsed DC, the resulting phenotype of the cell population was predominantly CD4+ Th2-like cells demonstrating high IL-4 production. The inclusion of anti-CD40 monoclonal antibody (mAb) to ligate CD40 on autologous DC induced both CD8+ and CD4+ T-cells that were specific for melanoma antigens. The CTL had high lytic activity against autologous tumour but not allogeneic tumour nor autologous fibroblasts. While many of the CD4+ T-cell clones were exclusively Th1 (IFN-γ+, IL-4-), many possessed a Th0 (IFN-γ+, IL-4+) phenotype. The clinical significance of these latter Th0 cells are currently unknown but newer findings in murine models of adoptive T-cells therapy suggest that better tumour eradication is achieved with both cell-mediated and humoral immunity [53].

DC-based T-cell expansion methods are an attractive approach because DC produce many of the necessary soluble activation factors for *ex vivo* expansion of antigen-specific T-cells. Several DC-based strategies are currently being developed for expansion including loading DC with apoptotic tumour cells, tumour antigen peptides, protein, or tumour cell-derived RNA. DC are the most potent antigen-presenting cells of the immune system and are responsible for initiating and modulating the immune response [54]. Unlike tumour cells, DC express co-stimulatory molecules and MHC class I and class II at high levels, and only a small number of DC are required to activate and expand tumour-specific T-cells.

The frequency of restimulation as well as the source of antigen appear to be important for determining the phenotype of the resulting T-cell population when using DC-based expansion. While repeated weekly stimulation of PBMC with renal cell carcinoma-loaded DC resulted in a predominant expansion of CD4+ T-cells, alternating between tumour cell loaded DC and irradiated tumour cells alone resulted in enriched

CD8+ T-cells with potent specificity for renal cell lines [55]. Preferential expansion of tumour-specific CD8+ T-cells can be accomplished by prior enrichment with reagents such as peptide MHC class I tetramers [56] before stimulation with tumour cell- or antigen-pulsed DC. DC can also be transfected with tumour-derived RNA as has recently been reported by Heiser and colleagues for the *ex vivo* generation of polyclonal T-cells against prostate and renal cell carcinoma [57,58].

Tumour cell or tumour RNA isolation can be avoided by transfecting or transducing DC with whole tumour antigen DNA, an approach that is attractive because it obviates the need to tailor make peptide antigens specific for the patient's MHC haplotype. This approach has been used by Bushenfelde and colleagues to generate CTL and Th cells specific for HER-2/neu using a retroviral transduction of DC [59]. DC were generated from CD34+ stem cells derived from patients and were transduced to express the full length HER-2/neu. Patient PBMC were then stimulated with the HER-2/neu-expressing DC. After 3 weekly stimulations, both CTL and Th cells could be individually cloned. The clones recognised different regions of the HER-2/neu suggesting the capacity of the DC to express multiple antigenic peptides. An important attribute of this approach is that the transduced DC do not express high levels of HER-2/neu but rather moderate to low levels, which would support the generation of high affinityT-cells which would in turn, be able to target tumour cells with a broad range of antigen expression [59].

While *ex vivo* expansion techniques can be optimised to produce T-cell lines with improved antigen-specificity and function, as assessed by *in vitro* assays, the ultimate goal is to carry those attributes into the host following transfer. The complex *in vivo* environment can present new challenges to the T-cells that may not have been present *in vitro*. For example, the immune microenvironment contains immunosuppressive factors which will inhibit the growth and function of transferred cells. Methods are being developed to improve the function of T-cells *in vivo* and to overcome these obstacles.

5. Enhancing *in vivo* function and longevity of transferred T-cells

In vivo supplementation with soluble T-cell growth factors can promote the activation and longevity of adoptive transferred T-cells. For example, murine models of adoptive T-cell transfer have demonstrated that administration of IL-2 following adoptive transfer maintains high levels of precursor specific for viral antigens for extended periods of time [1]. This is consistent with studies in IL-2 knockout mice where frequencies of transferred ovalbumin (OVA)-specific CD8+ T-cells declined significantly with time compared to the same T-cells injected into normal mice [60]. However, the toxicity of IL-2 in cancer patients limits its use and investigations are underway to identify less toxic strategies for the chronic maintenance of transferred T-cells. Recent findings suggest that iv. IL-2 alone may not be sufficient to extend the life of transferred melanoma-specific CD8+ T-cell clones, suggesting the need for additional factors [7]. Recent findings by the authors demonstrate that HER-2/neu-specific CD8+ T-cells can persist for at least a year when generated concurrently with HER-2/neu-specific CD4+ T-cells suggesting that co-infusion of antigen-specific CD4+ T-cells along with CD8+ T-cells during adoptive T-cell therapy may improve CD8+ T-cell longevity [21]. Alternatively, it would be beneficial to identify other pharmacological means of improving function and extending T-cell life *in vivo*.

One candidate receptor for *in vivo* modulation of infused T-cells is the OX-40 receptor (OX-40R). OX-40R is a transmembrane receptor expressed predominantly on activated CD4+ T-cells and is a member of the TNF receptor superfamily. The ligand for OX-40, OX-40L, is expressed on activated APC and B-cells. *In vivo* engagement of OX-40R with OX-40L during tumour priming results in enhanced tumour immunity through increased activation of the endogenous antitumour CD4+ T-cell response [61,62]. These responses were observed for a variety of murine tumours. Recently, OX-40R activation using OX-40 mAb has been applied to adoptive T-cell therapy of 10-day MC205 pulmonary metastases and intracranial tumours. In that study it was found that administration of OX-40R mAb resulted in the need for significantly fewer tumour-specific T-cells to cure established malignancy [63]. *In vivo* T-cell trafficking studies revealed that the OX-40R mAb application did not result in an increased number of T-cells trafficking to tumour sites suggesting that OX-40R stimulation results in enhanced function of tumour-specific T-cells.

Methods of genetic modification of T-cells to enhance cellular function, deliver therapeutic factors, or enhance T-cell longevity will likely play a key role in the success of adoptive T-cell therapy in tumour eradication [64]. The feasibility of these approaches has been demonstrated in the murine models, experimental autoimmune encephalitis (EAE) and non-obese mouse diabetes (NOD). It is clear that inflammatory Th1 CD4+ T-cells are pivotal in the development of both EAE and NOD [65,66]. Encephalitogenic or diabetegenic Th1 T-cells can be genetically modified to deliver immunosuppressive cytokines which can limit the extent of the disease [67]. For example, delivery of IL-4 by retrovirus-transduced encephalitogenic T-cells delays onset and reduces severity of EAE induced by immunisation against myelin basic protein [68]. Similarly, islet-specific Th1 lymphocytes, transfected to express IL-10, prevent adoptively-transferred diabetes in NOD mice [69]. These results demonstrate that tissue-specific T-cells can be altered genetically to skew the Th1/Th2 environment, ultimately changing the course of the autoimmune disease. The genetic alterations of T-cells specific for cancer would take reverse strategy. Tumour-specific T-cells could be manipulated to increase the Th1 type response at the site of tumour to further enhance inflammation. Target cytokines for overexpression might include IFN-γ, TNF-α and IL-2.

Tumour-specific T-cells can also be genetically engineered to control their *in vivo* growth capabilities. Since many human tumours grow slowly, treatment will likely require a sustained T-cell response to ensure eradication of all micrometastases as well as destruction of the primary tumour. Transferred T-cells, particularly CTL, are extremely short-lived in the absence of supplemental help and stimulation as previously discussed [7]. Aimed at improving the ability to manipulate the *in vivo* growth of tumour-specific T-cells, Evans and colleagues have developed a chimeric GM-CSF/IL-2 receptor which, when transduced into CTL, results in GM-CSF-sensitive proliferation mediated through the IL-2 signalling mechanism [70]. It is envisioned that systemic delivery of GM-CSF could be used to expand only transduced T-cells while avoiding the toxicities associated with IL-2 administration. In addition, the receptor is constitutively expressed and subject to local autocrine activation within the immune microenvironment.

Redirecting T-cell antigen-specificity is also possible using several different methods. One method is to transfect a TCR with known specificity into naive T-cells. As an example, Calogero and colleagues transduced Jurkat T-cells to express an HLA-A2-restricted αβ-TCR gene specific for a MAGE 3 peptide [71]. The resulting T-cell line was activated in response to both T2 cells and a melanoma cell line loaded with MAGE 3 peptide. A limitation of this technique is that in many cancers, TCRs and antigens are not adequately defined. However, in cases where antigens and TCRs are known, this technique, potentially, could be applied to naive non-specific T-cells following inactivation of their endogenous TCR genes.

Another retargeting method is to produce signalling receptors containing an extracellular antigen-specific antibody fused to an intracellular domain that is able to mediate T-cell activation. In one recent study, chimeric receptors containing an extracellular antibody and the Fc receptor γ signalling chain have been made against the colon cancer-associated protein EGP40 [72]. This chimeric receptor, GA733, when transduced into human T-cells conferred both cytokine production and cytolytic activity against EGP40+ colon cancer cells. In another study, Rossig and colleagues recently reported the generation of a T-cell line with dual recognition for EBV antigens and the neuroblastoma ganglioside antigen, GD2 [73]. EBV-specific T-cell lines were transfected with a construct containing the TCR-ζ-chain fused to variable domains of an anti-GD2 antibody. The resulting cells could potentially be maintained *in vivo a*nd *in vitro* with autologous EBV-infected cells.

In addition to enhancing T-cell function, immunosuppression may need to be circumvented during adoptive T-cell therapy. Infused tumour-specific T-cells, like endogenous tumour-specific T-cells, are likely targets of active systemic immunosuppression. Although multiple mechanisms of immunosuppression have been identified, recent investigations have focused extensively on T-regulatory cells (Tregs) [74-78], which play a key role in the maintenance of immune tolerance to self antigens. Tregs constitute a homogenous population of CD4+ CD25+ T-cells and are selected for in the thymus by self peptides *via* high affinity TCRs [79]. Tregs represent up to 6% of circulating PBMC in humans [75-77]. Selection of Tregs represents an alternative to clonal deletion of T-cells with high affinity TCRs against self antigens. Tregs directly inhibit the growth and function of antigen-specific T-cells by direct cell-to-cell contact [75-77], and recent findings suggest that Tregs proliferate in response to IL-2 in the absence of TCR stimulation. In addition to a direct suppressive effect on T-cells, Tregs have also been shown to down-modulate the co-stimulatory molecules, CD80 and CD86, on APC [80].

Studies in murine cancer models have suggested a role for Tregs in mediating evasion of tumour cells from immune destruction. Onizuka and colleagues demonstrated that rejection of tumour could be enhanced against 6/8 different murine tumours, including leukaemia, myeloma and sarcomas by depleting Tregs[81]. In that study, tumours were rejected in mice that had been previously treated with a single bolus dose of anti-CD25 antibody 4 days earlier. The injections of anti-CD25 antibody resulted in significantly reduced levels of circulating CD25+ T-cells. Similar findings were reported by Shimizu and colleagues who demonstrated that depletion of CD25+ T-cells results in the endogenous generation of antitumour immunity that is directed against a broad spectrum of tumours [82]. These encouraging findings that Tregs may play a key role in suppressing endogenous murine antitumour immunity have led investigators to examine the role of Tregs in mediating evasion of human tumours. Woo and colleagues recently reported evidence for increased levels of Tregs associated with both non-small cell lung cancer (NSCLC) and late-stage ovarian cancer [78]. Compared with autologous peripheral blood T-cells, there was an increased number (approximately 1.5 - 2.0-fold) of CD25+ CD4+ T-cells associated with either the tumour-infiltrating lymphocytes of NSCLC or tumour-associated lymphocytes of ovarian cancer. The CD25+ CD4+ T-cells were isolated and examined for cytokine release and found to secrete an immunosuppressive cytokine, TGF-β. Overcoming the immunosuppressive properties of these cells by direct inhibition or by swamping the effects of Tregs by a variety of mechanisms could be an important objective in designing therapeutically effective adoptive T-cell transfer. In the same study, it was also observed that the ovarian cancer patients, but not the NSCLC patients, had increased levels of CD25+ CD4+ T-cells within the peripheral blood compartment suggesting that strategies may need to be designed to neutralise suppressor activity during *ex vivo* expansion if using PBMC as the source of T-cells.

In addition to Tregs, other cells of haematopoietic origin have been identified that can suppress immunity. Defects in maturation pathways of dendritic cells lead to the accumulation of immature myeloid cells (ImC) in most cancer patients to levels 5 times higher than that observed in normal healthy individuals (< 3%) [83]. ImC, like Tregs, when added to cultures significantly inhibit antigen-specific T-cell proliferation. ImC can be induced to differentiate to mature DC in the

presence of all-*trans*-retinoic acid which simultaneously reduces their ability to inhibit T-cell function. It appears that the accumulation of ImC is a direct result of tumour presence since surgical removal of tumour can at least partially reverse these defects. The identification of suppressive haematopoietic T-cells in cancer patients is important and suggests that adoptive T-cells therapy, as well as tumour antigen-specific *ex vivo* expansion, can be improved through prior depletion of either Tregs or ImC.

6. Applications of imaging to immunotherapy

Recently developed biochemical imaging methods, in particular PET, play an increasing role in clinical oncology [84]. PET imaging may be helpful in the development of adoptive immunotherapy, in particular in three areas:

- monitoring tumour response to treatment
- tracking T-cell homing
- measuring the heterogeneity of antigen expression

The standard approach to measuring tumour response is to look for a decrease in tumour size as tumour cells die and the tumour mass shrinks [85]. However, size changes can lag cellular responses by weeks to months and for cytostatic therapies, tumour size may not change at all. It would therefore be logical to use biochemical imaging to look for evidence of tumour response earlier in the course of treatment. In this regard, PET imaging using [F-18]fluorodeoxyglucose (FDG) has shown that tumour glucose metabolism can decline early in response to successful cytotoxic chemotherapy, well in advance of changes in tumour size [86,87]. For immunotherapy, because a local immune response at the tumour site may be energy requiring, it may be difficult to evaluate tumour response by FDG PET alone. Recent work with PET tracers of cellular proliferation, such as [C-11]thymidine, has shown that measuring tumour cellular proliferation provides an early and quantitative estimate of tumour response to cytotoxic chemotherapy [87,88] and that the early changes in cellular proliferation in response to therapy are larger and more consistent than changes in glucose metabolism [87]. The combination of glucose metabolism and cellular proliferation measurements using PET, over the course of immunotherapy, will provide unique insights into the mechanisms and timing of the immune response *in vivo*.

To date, measuring the response of tumours to adoptive T-cell therapy has been limited to techniques that are restricted in their ability to quantify real time *in vivo* T-cell trafficking and homing. Indirect methods of monitoring therapy such as limiting dilution assays and ELISPOT can underestimate the number of antigen-specific T-cells at the tumour site, while more direct methods such as tetramer complexes are restricted to select MHC molecules. The radiolabelling of T-cells to allow *in vivo* trafficking offers promise in understanding tumour-host interactions, T-cell expansion and T-cell homing during adoptive T-cell therapy. In addition to non-specific labelling of *ex vivo* expanded T-cells, molecular imaging approaches targeting the expression of specific genes using reporters designed to work with particular PET tracers [89] may be able to take advantage of T-cell markers specific for different T-cell subsets with unique functional phenotypes. Imaging the homing of T-cells to tumours and regional lymph nodes can provide new insight into tumour-host interactions. In the setting of adoptive T-cell therapy, these methods can elucidate the functional interaction of different T-cell subsets with tumour cells and other effector arms of the immune system.

One potential explanation for the lack of success with early trials of antigen specific T-cell therapy is the development of tumour antigen-loss variants. Tumours may escape recognition by adoptively transferred T-cells by downregulation of antigens resulting in a tumour that can evade detection and destruction. Measuring the heterogeneity of antigen expression using radiolabelled antibodies may help identify alterations in antigen expression as a cause of therapy failure. This can be done using the single-photon emitting isotope ^{131}I, as in the recently described approach which predicted Herceptin efficacy and cardiotoxicity [90]; or possibly with the positron-emitter, [124]I, to provide a more quantitative measure of antigen expression than can be achieved using ^{131}I and conventional radiotracer imaging.

The application of PET imaging technology to measuring tumour metabolism and proliferation, T-cell trafficking and antigen expression in adoptive T-cell therapy may yield insights into the mechanisms underlying functional adoptive immunotherapy and host-tumour interactions. Such studies are currently underway in the authors laboratory and others. Learning more about the functional interactions between T-cells and tumour cells will enable the development of more specific and long lasting responses to tumours and ultimately improve the efficacy of adoptive T-cell therapy.

7. Conclusion

Evidence from both mouse experiments and human clinical trials suggests that the most effectiveT-cell populations will be those that aim to reconstitute full T-cell immunity representing both major T-cell subsets, CTL and Th. In order to generate an adequate T-cell population, *ex vivo*, appropriate culture conditions must be established and may be different for each individual T-cell subset or antigen-presentation system. A number of cytokines have been cloned and produced that can have important effects in culture when used with IL-2. Furthermore, novel methods of antigen-presentation have been established to facilitate *ex vivo* expansion. Recent progress in molecular biology has led to the development of more efficient methods for cloning, altering and transfecting T-cells in order to improve important T-cell characteristics such as antigen-specificity and *in vivo* longevity. Finally, the implementation of *in vivo* imaging using PET should provide a greater understanding of those characteristics that T-cells must possess in order to effectively home to and eradicate tumour.

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