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Adoptive immunotherapy for cancer: building on success

Luca Gattinoni[*], **Daniel J. Powell Jr.**[*], **Steven A. Rosenberg**, and **Nicholas P. Restifo**

Abstract

Adoptive cell transfer after host preconditioning by lymphodepletion represents an important advance in cancer immunotherapy. Here, we describe how a lymphopaenic environment enables tumour-reactive T cells to destroy large burdens of metastatic tumour and how the state of differentiation of the adoptively transferred T cells can affect the outcome of treatment. We also discuss how the translation of these new findings might further improve the efficacy of adoptive cell transfer through the use of vaccines, haematopoietic-stem-cell transplantation, modified preconditioning regimens, and alternative methods for the generation and selection of the T cells to be transferred.

Substantial progress has been made in our understanding of the molecular and cellular bases of T-cell-mediated antitumour responses. $CD8^+$ T cells have been identified as potent effectors of the adaptive antitumour immune response[1,2]. The target antigens that are recognized by tumour-reactive $CD8^+$ T cells have been shown to be mostly non-mutated self-antigens that are also expressed by tumour cells (and these antigens are denoted here self/tumour antigens) [1,2]. Tumour-specific $CD4^+$ T cells have been also identified, but their functionality can be manifold because $CD4^+$ T cells can help or hinder anti-tumour immune responses[3–5]. The molecular signals that modulate T-cell activation, function and memory are currently being elucidated. Both positive and negative signals from co-stimulatory molecules have been shown to shape the antitumour response[6,7]. Cytokines, including those signalling through receptors that contain the common cytokine-receptor γ-chain (γ_c), have been shown to alter the programming of effector $CD8^+$ T cells[8,9].

Despite a wealth of knowledge relevant to basic aspects of tumour immunology, the clinical realization of effective therapeutic vaccines for solid tumours has not yet been convincingly achieved. Enthusiasm about the effectiveness of cancer vaccines has often been grounded in surrogate and subjective endpoints, rather than reliable objective cancer regressions using standard oncological criteria. In a recent review of 1,306 vaccine treatments, including those conducted in the Surgery Branch at the National Cancer Institute (NCI), Maryland, USA, a

Correspondence to L.G. and N.P.R. e-mails: gattinol@mail.nih.gov; restifo@nih.gov.
[*]These authors contributed equally to this work. National Cancer Institute, National Institutes of Health, Mark O. Hatfield Clinical Research Center, Room 3-5762, 10 Center Drive, Bethesda, Maryland 20892-1201, USA.

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DATABASES
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http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene γ_c | CD4 | CD8 | CD25 | CD28 | CD62L | CCR7 | FOXP3 | IL-2 | IL-7 | IL-15 | IL-7α | IL-15α

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3.3% overall objective response rate was observed[10]. Results such as these highlight the need to improve current cancer vaccines[11] and to develop alternative immunotherapeutic strategies for the treatment of metastatic cancer[10].

Cancer vaccines aim to stimulate the adaptive arm of the immune system directly *in vivo*. 'Active immunotherapy' with therapeutic vaccines has been attempted despite the presence of many redundant negative influences of the host immune system[5,12] and tumour microenvironment[13,14]. By contrast, adoptive cell transfer (ACT) therapies achieve T-cell stimulation *ex vivo* by activating and expanding autologous tumour-reactive T-cell populations to large numbers of cells that are then transferred back to the patient[15–17]. Early attempts of ACT therapies using tumour-infiltrating lymphocytes (TILs) and immunoreplete patients met with some success[18]. However, previous preclinical studies indicated that immune ablation is an effective preconditioning regimen that can increase T-cell responses after adoptive transfer[19,20]. We have now reported that adoptive transfer of TILs after non-myeloablative, but lymphodepleting, systemic chemotherapy (FIG. 1) can induce clear and reproducible responses in a substantial percentage (~50%) of patients[21,22]. Notably, dramatic tumour regressions can be elicited in patients with multivisceral, bulky melanoma that is refractory to standard treatments including chemotherapy, radiation and cytokine therapies (FIG. 2).

Here, we describe the mechanisms by which the transfer of tumour-reactive T cells into a lymphopaenic recipient mediates tumour regression, and the phenotypic and functional characteristics of tumour-specific T cells that induce antitumour responses *in vivo*. These factors provide the bases for rational design of new ACT-based immunotherapies that incorporate vaccines, increased intensity preconditioning regimens with haematopoietic stem cell (HSC) transplantation, and alternative methods for the generation and selection of T cells for transfer.

Lymphodepletion increases the efficacy of ACT

It has long been observed in mice that depletion of immune cells before ACT can markedly improve the antitumour efficacy of transferred $CD8^+$ T cells[19,20], but the specific mechanisms that contribute to this increased immunity have only recently begun to be elucidated. Although it seems counter-intuitive that the efficacy of ACT-based tumour immunotherapy can be improved by the removal of the host immune system, several mechanisms might underlie the augmented efficacy of tumour-reactive T cells in the lymphopaenic environment. These factors include the elimination of immunosuppressive cells such as $CD4^+CD25^+$ regulatory T (T_{Reg}) cells, the depletion of endogenous cells that compete for activating cytokines, and the increased function and availability of antigen-presenting cells (APCs) (FIG. 3).

Elimination of immunosuppressive cells

T_{Reg} cells are crucial for the maintenance of peripheral self-tolerance and for the suppression of antitumour responses[5]. T_{Reg} cells represent a unique T-cell lineage that is characterized by expression of the transcription factor forkhead box P3 (FOXP3) and high levels of expression of cell-surface molecules associated with T-cell activation, including CD25 (also known as IL-2Rα), glucocorticoid-induced tumour-necrosis factor (TNF)-receptor-related-protein (GITR) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)[5]. However, exclusive molecular signatures for human T_{Reg} cells do not currently exist because activation of $CD4^+$ T cells can also result in upregulation of FOXP3 expression[23]. Experiments using mice lacking T_{Reg} cells, owing to specific gene defects, as well as the 'add-back' of these cells, have convincingly shown that they suppress the antitumour activities of adoptively transferred self/tumour-reactive T cells[24]. Augmented antitumour responses were observed after ACT of self/tumour-reactive effector $CD8^+$ T cells to tumour-bearing $Cd4^{-/-}$, but not $Cd8^{-/-}$, mice, indicating that the immunoregulatory cells are contained in the $CD4^+$ T-cell population. The

suppressive activity was restricted to the $CD25^+$ T-cell subset because transfer of $CD4^+CD25^+$ T_{Reg} cells alone, or in combination with $CD4^+CD25^-$ T helper (T_H) cells, inhibited effective immunotherapy in lymphopaenic mice. By contrast, transfer of T_H cells alone resulted in profound autoimmunity and eradication of established tumour. Interstingly, the maintenance and function of effector $CD8^+$ T cells required the presence of T_H cells that were able to produce interleukin-2 (IL-2)[24].

The immunosuppressive role of T_{Reg} cells in patients with cancer has only recently begun to be explored. T_{Reg} cells, which are over-represented in tumour lesions from patients with melanoma and lung cancer, can inhibit the function of infiltrating T cells[25,26], and T_{Reg} cells specific for melanoma antigens have been described[4]. Reduced survival was reportedly associated with increased tumour infiltration by T_{Reg} cells in patients with ovarian cancer[27], although these findings have been recently contradicted[28]. Therefore, at present, no conclusive data link the *in vivo* function of T_{Reg} cells and the progression of cancer. Nevertheless, the suppressive effects of T_{Reg} cells might contribute to the poor clinical response rates reported in patients with cancer who receive immunotherapy in non-lymphodepleting settings. Selective elimination of T_{Reg} cells[29] from TILs of patients might further improve the efficacy of ACT approaches in the lymphodepleting setting, because T_{Reg}-cell proliferation can be increased by the lymphopaenic environment and the presence of exogenous IL-2 (REFS 30,31). Furthermore, removal of T_{Reg} cells from peripheral-blood lymphocytes (PBLs) might generate a population of cells that is enriched for T_H cells that are able to bolster the response of self/tumour-specific $CD8^+$ T cells *in vivo*[24].

Other immune cells, including natural killer (NK) cells, natural killer T (NKT) cells and $CD11b^+Gr1^+$ myeloid suppressor cells (MSCs), have been shown to dampen T-cell function[32–34]. Little is known about the immunosuppressive activities of NK and NKT cells, although a perforin-dependent immunosuppressive mechanism has recently been reported for NK cells[33]. More is known about MSCs, which are a heterogeneous population of cells that comprises myeloid cells at various stages of differentiation, including monocytes, granulocytes and a subset of immature myelo-monocytic cells[34]. Increased frequencies of MSCs are found in conditions characterized by impaired T-cell function, including tumours and chronic infections[34]. In mice and humans, MSCs can infiltrate the tumour bed and inhibit T-cell responses through mechanisms involving direct contact with tumour-reactive T cells, L-arginine consumption and the release of L-arginine metabolism products[35,36]. Depletion of MSCs using a Gr1-specific antibody can result in protection from tumour challenge[37]. Therefore, removal of MSCs, and thereby their suppressive activity, might contribute to the increased antitumour T-cell responses observed after ACT in patients that have been lymphodepleted.

Minimizing cellular cytokine sinks

Transfer of small numbers of antigen-specific T cells into a lymphopaenic host results in the expansion and activation of the transferred T-cell population, a process that is known as homeostatic proliferation[38–40]. In the lymphopaenic environment, antigen-experienced T cells proliferate independently of self-peptide–MHC complexes[40]. However, either co-transferring an ‘irrelevant’ population of T cells or increasing the number of transferred cells can reduce the level of homeostatic proliferation in a dose-dependent manner, showing that other elements exist that limit homeostatic proliferation[39–41]. Although host-mediated inhibition of the proliferation of adoptively transferred T cells might involve direct cellular contact, competition might also exist between transferred and host T cells for a limited amount of the cytokines that are required to support $CD8^+$ T-cell homeostasis; such competition is known as the ‘cytokine sink’ effect[12,42].

The importance of the availability of these cytokines has been shown in experiments in which mice deficient for IL-7 or IL-15 showed impaired homeostatic maintenance and proliferation of memory $CD8^+$ T cells[43–45]. Conversely, transgenic mice overexpressing IL-7 or IL-15 have increased numbers of T cells, owing to the preferential expansion of the memory $CD8^+CD44^{hi}$ T-cell population[46,47]. In the pmel-1 mouse model of ACT therapy[48], lymphodepletion before cell transfer increased the persistence of self/tumour-specific T cells, as well as their effector function and tumour regression, compared with immunoreplete hosts[42,49]. In mice deficient for both IL-7 and IL-15, the beneficial effect of ablation was completely abrogated[42]. Conversely, increased antitumour responses were seen when these cytokines were exogenously administered and when the host lymphocytes competing for these cytokines were removed by using mice lacking both recombination-activating gene 2 (*Rag2*) and γ_c[42]. These findings show that a key mechanism underlying the improved efficacy of ACT therapies after lymphodepletion is the transient eradication of endogenous lymphocytes, which serve as cellular cytokine sinks[42].

Elucidating the specific endogenous cellular components that function as cytokine sinks is important for understanding the mechanism by which lymphodepletion augments the effectiveness of ACT-based therapies. In *Rag1*$^{-/-}$ mice (which, unlike *Rag2*$^{-/-}\gamma_c^{-/-}$ mice, lack B cells and T cells but do have NK cells), more extensive tumour regression was observed in irradiated recipients than in non-irradiated recipients, whereas in *Rag2*$^{-/-}\gamma_c^{-/-}$ hosts, ACT treatment became so efficacious that it was difficult to detect the effects of irradiation[42]. This finding, coupled with the observation that depletion of cells expressing NK1.1 (using an NK1.1-specific antibody) improves the efficacy of ACT therapy in tumour-bearing *Rag1*$^{-/-}$ mice[42], implicates NK cells as key effectors of the cytokine sink effect, a process that might be mediated by consumption of IL-15, a crucial cytokine for the survival and proliferation of NK cells *in vivo*[50,51].

IL-2, another cytokine that signals through a receptor containing γ_c, is a T-cell growth factor that is commonly used to promote the expansion and function of tumour-specific T-cell populations *in vitro* and *in vivo*[15]. Perhaps more importantly, IL-2 is essential for the maintenance of peripheral self-tolerance[52]. Mice deficient in either IL-2 or components of the IL-2 receptor spontaneously develop lymphoproliferative and autoimmune disorders[52]. These observations have been linked to impaired T_{Reg}-cell homeostasis and 'metabolic fitness' *in vivo*, rather than suppressive function, because T_{Reg} cells from either IL-2- or IL-2Rα (CD25)-deficient mice are competent when tested in *in vitro* assays of suppressive function[53]. However, more recent findings have shown that *in vivo* IL-2 signalling is important not only for maintaining T_{Reg}-cell 'fitness' but also for their suppressive function[54,55]. Antitumour activity of adoptively transferred self/tumour-specific $CD8^+$ T cells was inhibited in wild-type, but not IL-2Rα -deficient, mice despite both having comparable numbers of $FOXP3^+$ T_{Reg} cells[54]. Furthermore, blockade of IL-2Rα with specific antibody can induce profound autoimmunity resulting from impaired T_{Reg}-cell function, rather than depletion of these cells[55]. These results indicate that *in vivo* immunoregulation by T_{Reg} cells might, in part, be a product of their constitutive expression of the component of the IL-2 receptor that confers the highest affinity for IL-2, IL-2Rα , and their increased capacity to consume IL-2 (REFS 54,56). Therefore, removal of T_{Reg} cells by lymphodepletion might result in increased antitumour reactivity of adoptively transferred $CD8^+$ T cells, not only by the elimination of direct cellular inhibition but also through increased availability of IL-2.

Improved APC function and availability

Systemic chemotherapy and total body irradiation have both been used before ACT to deplete the lymphoid compartment of the host and create a niche for the transferred cells. Investigators have long hypothesized that these treatments might also cause necrosis or apoptosis of tumour

cells, resulting in APC uptake of tumour antigens and the subsequent cross-presentation of these antigens to the adoptively transferred tumour-reactive $CD8^+$ T cells[57]. Although lymphodepletion can reduce the absolute number of APCs *in vivo*, it can also promote their transition to an activated state[58,59]. In mice, the expression of the activation markers CD86 and $I\text{-}A^b$ (an MHC class II molecule) has been reported to be upregulated on the surface of splenic dendritic cells (DCs) after irradiation[59]. Furthermore, DCs that were isolated after irradiation released substantially more IL-12 than DCs that were isolated from non-irradiated mice[59]. Activation of DCs after chemotherapy or irradiation might be triggered by translocation of bacterial products, such as lipopolysaccharide (LPS) and other Toll-like receptor (TLR) agonists, into the blood following damage to the integrity of mucosal barriers[60]. The production of pro-inflammatory cytokines such as TNF, IL-1 and IL-4 by host cells might also be involved in mediating DC maturation[58,61–63]. In addition, the lymphopaenic environment might facilitate the activation of transferred self/tumour-reactive T cells through decreased competition at the surface of antigen-bearing APCs[64]. Although the net effect of lymphodepletion on APC function is less clear than its impact on T_{Reg} cells and cellular cytokine sinks, ablation might ultimately increase the antitumour reactivity of transferred T cells by increasing the activation and availability of APCs.

T-cell differentiation state and ACT

Lymphodepletion can have a marked impact on treatment with ACT-based immunotherapies, but it is not the only factor responsible for affecting clinical responses. Emerging findings from both mouse studies and clinical trials indicate that intrinsic properties related to the differentiation state of the adoptively transferred T-cell populations are crucial to the success of ACT-based approaches[65–68].

$CD8^+$ T-cell subsets in both mice and humans can be categorized into distinct differentiation states on the basis of phenotypic and functional attributes[69,70] (FIG. 4). A progressive pathway of $CD8^+$ T-cell differentiation[69,70] has gained acceptance based on the findings of *ex vivo* phenotypic analyses of virus-specific T cells[69], measurement of telomere length[71], gene-expression profiling[72] and *in vitro* differentiation studies[65,71]. Within this model, activation of naive $CD8^+$ T cells results in proliferation and progressive differentiation through early, intermediate and late effector stages depending on signal strength[70] (FIG. 4). Memory $CD8^+$ T cells might reflect T cells arrested at intermediate stages of the differentiation pathway[73,74], but there remains some debate regarding the pathways by which effector and memory T cells develop[75].

The phenotypic and functional characteristics of self/tumour-specific $CD8^+$ T cells that are associated with optimal *in vivo* tumour responses in the pmel-1 mouse model of ACT therapy have recently been elucidated[65]. Self/tumour-specific $CD8^+$ T cells at progressive stages of differentiation were generated using multiple *in vitro* stimulations with antigen. Surprisingly, $CD8^+$ T cells that acquired terminal effector properties and had increased antitumour activity *in vitro* were found to be less effective at triggering tumour regression *in vivo*. Terminally differentiated $CD8^+$ T cells were nearly 100-fold less effective *in vivo* on a per-cell basis than T cells at an early stage of differentiation. Similar findings have been reported by other groups using different mouse tumour[76,77] and allogeneic HSC transplantation[78,79] models. *In vitro* expansion of T cells for ACT — as it is currently performed for clinical use — induces progressive $CD8^+$ T-cell differentiation towards a late effector state, resulting in phenotypic and functional changes that make T cells less ‘fit’ to mediate antitumour responses *in vivo* and less able to benefit from the activating cues present in the lymphopaenic host (FIG. 4). For example, less-differentiated, central-memory-like T cells have a high proliferative potential, are less prone to apoptosis than more differentiated cells and have a higher ability to respond to homeostatic cytokines, ,ecause they express receptors such as the IL-7 receptor α -chain

(IL-7Rα)[65,75,80,81]. Therefore, less-differentiated, central-memory-like T cells might proliferate and become fully activated in the lympho paenic environment, which is rife with homeostatic cytokines such as IL-7.

Similar to mouse studies, ACT of human tumour-reactive $CD8^+$ T-cell clones that were generated and expanded *ex vivo* through multiple stimulations did not meditate objective responses in either immunoreplete[16] or immunodepleted patients[82]. T-cell clones used for therapy were highly avid and showed potent tumour-specific cytolytic activity *in vitro*, but they did not persist after infusion, indicating that they were in a state of terminal differentiation[16,82] (FIG. 4).

The importance of trafficking to lymph nodes

Tumour immunologists have long sought to cause T cells to specifically traffic to their tumour targets[83,84]. The loss of expression of the lymphoid homing molecule CD62L and the acquisition of CD44 expression were reported to be associated with increased antitumour effects of adoptively transferred T cells[76,85]. However, it is now clear, in both tumour and viral models, that T cells that can home to secondary lymph nodes, where they can be effectively stimulated by DCs, are more effective in adoptive immunotherapy[65,75,81]. Indeed, tumours alone are inefficient at triggering effective immune responses[65,81,86]. Antitumour responses were abrogated in hosts devoid of peripheral lymphoid tissues and with a disrupted splenic structure[81]. Furthermore, after transfer, CD62L-deficient self/ tumour-reactive $CD8^+$ T cells were markedly impaired in their ability to inhibit tumour growth compared with CD62L-sufficient T cells[65,81]. Similarly, CD62L-deficient T cells were less effective at mediating alloresponses in an allogeneic HSC transplantation model[78]. Therefore, downregulation of expression of lymph-node homing molecules at the intermediate and late stages of effector $CD8^+$ T-cell differentiation can result in impairment of their antitumour capacity. However, the principle that T cells must home to lymph nodes to be effective has not been established in humans. Despite the lack of expression of lymph-node homing molecules, adoptively transferred $CD62L^-CCR7^-$ TILs[87] were able to engraft, proliferate and ultimately induce objective responses in ~50% of patients[21,22].

Co-stimulatory molecules and T-cell persistence

Transition from an early to an intermediate effector stage is marked by downregulation of CD28 expression. Interaction of CD28 with CD80 and/or CD86 on APCs amplifies T-cell receptor (TCR)-mediated T-cell activation and proliferation[88]. Secretion of IL-2, induction of anti-apoptotic molecules and accelerated cell-cycle progression have been reported for CD28-expressing T cells[88,89]. The role of CD28 expression in ACT-based clinical trials has been recently investigated in detail. Tumour-specific TILs express low, but detectable, levels of CD28 (REF. 87). After cell infusion, immediate and high expression of CD28 was detected on circulating tumour-reactive T cells, indicating that either rapid upregulation of CD28 expression or early selective expansion and survival of the $CD28^+$ T-cell population had occurred. Analysis of persisting and non-persisting TIL clones indicates preferential survival of the clonotypes expressing the highest levels of CD28, implicating a survival advantage for transferred T cells with an early effector phenotype[67,68].

Engagement of the co-stimulatory molecule CD27 can also augment TCR-induced T-cell proliferation and is required for the generation and maintenance of memory T cells *in vivo*[90,91]. Consistent with a late effector state, T cells lacking CD27 have been shown to have potent cytolytic function and secrete little IL-2 (REF. 75). In the pmel-1 mouse model, self/ tumour-specific late effector cells were less effective at mediating tumour regression after adoptive transfer relative to early effector T cells that express high levels of CD27 (REF. 65). Moreover, the administration of soluble CD27 ligand, CD70, augmented *in vivo* $CD8^+$ T-cell

responses to viral infection and tumour challenge by increasing the expansion and maintenance of the antigen-specific T-cell population, indicating that CD27 expression is not only a marker of less-differentiated T cells but also functionally crucial for optimal immune responses[92]. In the clinical arena, a statistically significant difference in the frequency and number of CD27-expressing cells could be found in bulk TIL populations from responding versus non-responding patients when IL-2 was withdrawn[93]. After ACT, the frequency of TILs expressing CD27 gradually increased and was associated with the long-term maintenance of stable numbers of tumour-specific T cells in responding patients[87]. This result, and findings from viral studies, predicts that T cells that express CD27 selectively persist *in vivo*, giving rise to a stable population of memory $CD8^+$ T cells[87,94].

Homeostatic cytokine signals and T-cell persistence

Increased access to homeostatic cytokines has been shown to be crucial for the enhanced antitumour responses that occur following ACT to lymphodepleted hosts[8,12,42]. Homeostatic signals can be regulated by both the availability of cytokines in the host and the level of expression of the cytokine receptors on the surface of transferred $CD8^+$ T cells. Expression of IL-7Rα by a subset of effector $CD8^+$ T cells might identify precursors that are destined to become long-lived memory cells[80]. IL-7Rα ^{low} self/tumour-specific late effector $CD8^+$ T cells transferred to tumour-bearing mice persisted at decreased numbers and were less effective at inducing antitumour responses than were IL-7Rα ^{hi} early effector $CD8^+$ T cells[65]. In patients, IL-7Rα was expressed at low levels on all TIL populations at the time of ACT, but it was upregulated immediately after infusion on the surface of robustly proliferating tumour-specific T cells that persisted[87]. Therefore, IL-7 signalling seems to be important for the immediate and long-term survival of tumour-specific T cells after ACT.

IL-15Rα was weakly expressed by most TILs used for ACT and, unlike IL-7Rα, was not upregulated on persisting tumour-specific T cells after ACT[87]. However, IL-15 signalling might remain intact because trans-presentation of IL-15 by APCs and stromal cells can occur[95].

T-cell persistence and antitumour response

Because IL-2 is provided both *in vitro* during expansion of T-cell populations and *in vivo* in the immediate aftermath of cell infusion, tumour-reactive $CD8^+$ T cells might undergo apoptosis after IL-2 withdrawal[96]. Because early effector T cells have the capacity to release IL-2, selective survival of these cells might occur in an autocrine fashion[65]. In addition, early effector T cells have survival advantages over intermediate and late effector T cells, as reflected by the expression of lower levels of the pro-apoptotic molecules BID (B-cell lymphoma 2 (BCL-2)-homology domain 3 (BH3)-interacting-domain death agonist), BAD (BCL-2-antagonist of cell death) and CD95L (CD95 ligand; also known as FASL), and higher levels of anti-apoptotic molecules[65,81]. The intrinsic proliferative capacity of adoptively transferred T cells might also affect their ability to engraft and persist. Increased proliferation of the early effector T-cell subset has been seen *in vitro* and *in vivo* following stimulation with cognate antigen[65]. In parallel with T-cell proliferation and progressive differentiation, gradual telomere erosion occurs until a critical degree of shortening (termed the Hayflick limit) results in chromosomal abnormalities, and cell death or senescence[97]. This process might be partially compensated for by telomerase activity[97]. Therefore, telomere length and telomerase activity can influence T-cell replicative capacity. Interestingly, recent analyses of human TILs have shown a correlation between the length of the telomeres of the transferred cells and persistence of T cells *in vivo* following ACT, indicating that in addition to tumour-antigen recognition, the intrinsic proliferative capacity of adoptively transferred T cells might also be a factor affecting persistence and successful tumour treatment[68].

Optimizing tumour-reactive T cells for ACT

The finding that less-differentiated, central-memory-like T cells might be the optimal population for ACT-based immunotherapies raises a clinical problem. Data from animal studies indicate a direct correlation between the number of adoptively transferred T cells and antitumour responses *in vivo*, leading to the idea that large numbers of tumour-reactive T cells must be administered to patients to obtain therapeutically effective antitumour responses[65,76]. Therefore, in clinical trials, tumour-reactive $CD8^+$ T-cell populations are expanded to large numbers *in vitro* with CD3-specific antibody plus IL-2 or with specific-antigen plus IL-2, which drives differentiation of T cells to intermediate and late effector stages of differentiation[16,22,82]. New findings in mice emphasize that the quantity of transferred T cells is an important factor when T cells with the same quality and fitness are being used for ACT. Increased antitumour responses were observed after adoptive transfer of low numbers of 'fit' (early effector) T cells compared with high numbers of 'unfit' (late effector) T cells[65,76]. Therefore, one of the greatest challenges in the field is currently the generation of large numbers of 'fit' T cells for ACT.

Modifications of current in vitro protocols for expanding T-cell populations

Using a standard rapid expansion protocol, TILs for transfer are selected and populations are expanded for about 2 weeks with CD3-specific antibody, high doses of IL-2 and irradiated allogeneic feeder cells[22] (FIG. 1). This procedure results in the differentiation of tumour-specific $CD8^+$ T cells to an intermediate and late effector state. Limiting the *in vitro* expansion phase to a short duration might markedly improve the 'fitness' of the transferred T cells because a greater percentage of tumour-reactive T cells express CCR7, co-stimulatory molecules and IL-7Rα , and are actively dividing in the first week of growth[98]. The question remains whether this improved fitness can compensate for the reduced number of cells generated soon after activation.

Cytokines, acting in concert with signals through the TCR and co-stimulatory molecules, can function as accelerators or brakes for T-cell proliferation and differentiation[70]. IL-2 has been shown to be an effective T-cell growth factor but has undesirable effects, including the ability to decrease the expression of lymph-node homing molecules and to promote the terminal differentiation of T cells, predisposing them to activation-induced cell death[65,99]. Other cytokines that signal through a receptor that contains γ_c, such as IL-15, can analogously induce the *in vitro* expansion of tumour-reactive $CD8^+$ T-cell populations for ACT[8,65,100]. IL-15 supports the growth of similar numbers of T cells as IL-2, but it does not induce the detrimental T-cell differentiation and apoptosis that IL-2 does[65,101,102]. T-cell populations that had been expanded in the presence of IL-15 were shown to have a superior ability to elicit tumour regression *in vivo* after ACT to tumour-bearing mice, compared with T-cell populations that had been expanded in the presence of IL-2 (REFS 8,[65,81]). Other cytokines that signal through a receptor that contains γ_c (including IL-7 and IL-21) that were evaluated in a similar manner did not promote robust proliferation or differentiation of self/ tumour-reactive $CD8^+$ T cells *in vitro*, but they had a greater antitumour efficacy than IL-2 treated cells *in vivo* (Hinrichs C. S., unpublished observations). By contrast, no differences in the differentiation state of tumour-reactive T cells from vaccinated patients were detected when the cells were stimulated *ex vivo* with cognate antigen in the presence of IL-2, IL-7 or IL-15 (REF. 103). Results obtained using human cells probably reflect the use of antigen-experienced T cells that have already differentiated into intermediate and late effector stages, instead of the naive populations that are used in mouse studies[104]. Indeed, stimulation of naive human tumour-reactive T cells in the presence of IL-21 induced the preferential expansion of a less-differentiated $CD28^{hi}CD45RO^+$ T-cell population that could release IL-2 after stimulation with cognate

antigen[9]. Therefore, the ability to obtain naive tumour-specific $CD8^+$ T cells might be of paramount importance to improving current ACT-based therapies.

Genetic modification of T cells for ACT

Naturally occurring self/tumour-specific T cells have been described in patients with cancer, as well as in healthy individuals[105,106]. Antigen-experienced $CCR7^-CD45RA^-CD45RO^+$ self/tumour-specific T cells are preponderant in the metastatic lymph nodes and are uniformly present at tumour sites, whereas naive self/tumour-specific T cells are predominantly found in the blood[106]. Unfortunately, these naive cells are mainly characterized by a low TCR avidity, thereby making them unsuitable for ACT[107]. To circumvent this issue, high-affinity TCRs, derived from TILs that mediate strong *in vivo* tumour regression, have been identified, cloned and transduced into the PBLs of patients with cancer[108–110]. These TCR-transduced PBLs have cytolytic activity, secrete cytokines *in vitro* after stimulation with melanoma-cell lines and are currently being clinically evaluated[109,110].

The affinity of the TCR selected for transduction, the level of transduced TCR expressed on the cell surface and the differentiation state of the transduced T cells that are used for ACT might contribute to the success of trials following TCR transduction. Naturally occurring T cells that express high-affinity TCRs specific for self/ tumour antigens might be difficult to obtain owing to intrathymic deletion. However, high-affinity TCRs can be generated *in vivo* in immunized HLA-A2-transgenic mice[111,112] or *in vitro* by phage display of TCRs containing degenerate complementarity-determining regions[113].

Integration of retrovirally delivered sequences requires active division of target cells, a process that also promotes T-cell differentiation (FIG. 5a). As PBLs contain T cells at multiple stages of differentiation, inducing activation and proliferation of PBLs guarantees that TCR-transduced T cells are largely devoid of naive T cells. Alternatively, as lentiviral vectors are less dependent on active cell division, they might be used to transduce high-affinity TCRs into T cells without driving differentiation[114]. Lentiviral transduction of T cells that are pre-selected for specific markers might therefore be a way of generating large numbers of naive tumour-specific T cells for ACT (FIG. 5b).

Delivery of both the TCR α -chain (TCRα) and β-chain (TCRβ) directs the expression of the intact TCR; however, pairing with endogenous TCRα and TCRβ can occur, thereby reducing the surface density of tumour-specific TCR (FIG. 5a,b). Transduction of HSCs followed by T-cell-lineage differentiation — through *in vitro* Notch signalling[115,116] or through natural development *in vivo* in immunodeficient mice[117] — is an attractive approach to overcome this problem. Forced expression of transduced TCRs by differentiating HSCs facilitates the repression of expression of the *Rag* genes, such that endogenous TCRβ are not expressed[118] (FIG. 5c). Alternative approaches to overcome the problem of mispairing with endogenous TCRα and TCRβ might include the manipulation of the transmembrane-association domains of TCRα and TCRβ[119], the use of chimeric receptors with antibody specificity (known as T-bodies)[120] and TCR transduction into T cells that lack an α βTCR, such as γδ T cells[121].

Several other genes have been proposed for transduction of tumour-reactive T cells to improve their quality and functionality[122]. These include co-stimulatory molecules[89], anti-apoptotic molecules[123], pro-inflammatory or homeostatic cytokines[96,102] and chemokine receptors[84]. Although these manipulations are able to alter specific cell functions in differentiated tumour-specific T cells, the TCR approach confers self/tumour-specificity to cells that might have all the desired characteristics. Transduction with genes encoding TCRs specific for known epitopes allows the concurrent use of vaccines to potentiate the antitumour response of adoptively transferred T cells[124–126]. Another interesting approach includes the modulation of transcription factors such as BCL-6 (REFS 127,[128]), BCL-6B[129], lymphoid-

enhancer-binding factor 1 (LEF1) and T-cell factor 7 (TCF7)[130] in intermediate and late effector tumour-reactive T cells that might revert T cells to a less-differentiated state[131].

Concluding remarks

ACT to a lymphodepleted host has emerged as a promising advance in cancer immunotherapy. Preclinical and clinical studies have identified multiple mechanisms contributing to successful adoptive immunotherapies, including host-related factors, as well as the phenotypic and functional characteristics of the tumour-reactive T cells used for transfer. These findings provide the rationale for the design of new clinical protocols for the treatment of patients with cancer.

The improved effectiveness of immunotherapy following a non-myeloablative lymphodepleting regimen provides the rational basis for the evaluation of more intensive conditioning regimens such as a myeloablative regimen in conjunction with autologous HSC transplantation[132]. In the pmel-1 mouse model of ACT therapy, the use of a myeloablative regimen profoundly depleted host immunosuppressive cells and cellular sinks for activating cytokines, resulting in an increased ratio of effector cells to endogenous cells and increased anti-tumour responses compared with non-myeloablative conditioning (C. Wrzesinski, unpublished observations). The improved therapeutic effect was independent of antigen-specific vaccination but required the transfer of HSCs, which increased the proliferation and survival of co-administered self/tumour-reactive T cells, possibly through the release of cytokines, growth factors and anti-apoptotic factors (C. Wrzesinski, unpublished observations). The finding that myeloablative conditioning with a HSC transplant removed the need for specific vaccination has important implications for ACT-based immunotherapies in humans, which use polyclonal TILs for which the specificity is often unknown and for which effective vaccines are not currently available[10]. The use of a myeloablative preconditioning regimen involving chemotherapy and total body irradiation together with HSC transplantation in humans is currently under evaluation.

Increased immunity might be achieved with the use of more selective approaches to lymphodepletion to eliminate the toxicities associated with the use of non-specific preconditioning regimens based on chemotherapy and radiation. For example, T_{Reg} cells and other immunosuppressive cells might be selectively depleted with directed immunotoxins or suppressed by administering a cytokine such as TNF[133–136]. To overcome the sink effect of competing endogenous cells, saturating levels of activating cytokines might be provided exogenously[137]. Because IL-2 can promote T_{Reg}-cell proliferation and suppressive function, other cytokines that signal through a receptor that contains γ_c, such as IL-7, IL-15 and IL-21, might be preferable[12,42]. Alternatively, administration of IL-2–IL-2-specific antibody complexes could be used to selectively stimulate effector T cells rather than T_{Reg} cells[138]. Moreover, T_H cells that can produce many cytokines might be co-transferred with self/tumour-reactive T cells[24]. Finally, APCs might be activated through selective ligation of activation-associated molecules such as TLRs[139]. The use of combinatorial approaches might be of greater clinical benefit than single modality strategies.

Mouse models have now shown that early effector T cells mediate better *in vivo* antitumour responses than intermediate and late effector T cells on the basis of their increased proliferative and survival potential, receptiveness to homeostatic and co-stimulatory signals, homing to secondary lymphoid tissues and ability to secrete IL-2 (REF. 65). In humans, mounting evidence seems to support the preclinical finding that less-differentiated T cells are the ideal cells for ACT[66–68]. Taken together, these findings indicate that the current criteria for selection of T cells for ACT, including release of interferon-γ or cytolysis alone, are sub-optimal. Consideration of other important factors for selection such as phenotype, telomere

length, alternative cytokine production (such as IL-2) and TCR affinity are currently being investigated. The next generation of ACT-based immunotherapies might rely on the ability to endow 'fit' cells with elevated cell-surface expression of high-affinity, self/tumour-specific TCRs by gene-transfer technology that can be used in conjunction with specific vaccines[48, 124–126]. Ultimately, the TCR gene-therapy approach might hold the key to the widespread application of ACT-based therapy to the treatment of cancers of multiple histologies[110,112].

Note added in proof

It has recently been shown that T cells also express the transcriptional repressor B-lymphocyte-induced maturation protein 1 (BLIMP1)[140,141]. This provides a further potential transcription-factor target to modulate in an attempt to generate less differentiated T cells.

Common cytokine-receptor γ-chain

(γ_c). A signalling subunit of the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21.

Standard oncological criteria

Clinical criteria that determine whether or not a treatment for cancer is effective. The World Health Organization originally defined an objective clinical response as a 50% decrease in the sum of the products of perpendicular diameters of all lesions without an increase greater than 25% in any lesions or appearance of new lesions. Subsequent updated criteria are known as response evaluation criteria in solid tumours (RECIST). RECIST defines an objective clinical response as a 30% decrease in the sum of the longest diameters of target lesions, without an increase greater than 20% in any target lesions or appearance of new lesions.

Tumour-infiltrating lymphocytes

(TILs). The heterogeneous population of T cells found in a tumour bed. These cells are characterized by a diversity of phenotypes, antigen specificities, avidities and functional characteristics. They can be activated and expanded *ex vivo* and re-infused into the tumour-bearing host.

Non-myeloablative regimen

Treatment that induces a severe, but transient, leukopaenia without permanent damage to haematopoietic stem cells, thereby allowing spontaneous recovery of the haematological function of the host.

Homeostatic proliferation

A process of activation and proliferation of leukocytes in the lymphopaenic environment. T-cell homeostatic proliferation is driven by T-cell receptor interactions with self-peptide–MHC complexes and T-cell responsiveness to cytokines such as interleukin-7 (IL-7), IL-15 and possibly IL-21.

Pmel-1 mouse model of ACT

A mouse model of adoptive cell transfer (ACT) therapy for established B16 melanomas and autoimmunity against the melanocyte-associated differentiation antigen gp100. Treatment consists of adoptive transfer of gp100-specific $CD8^+$ T cells derived from the T-cell receptor (TCR) transgenic mouse pmel-1 in combination with altered ligand vaccine and cytokines that signal through a receptor that contains the common cytokine-receptor γ-chain (γ_c).

Cross-presentation

The process whereby antigen-presenting cells take up, process and present extracellular antigens, in association with MHC class I molecules, to $CD8^+$ T cells.

Toll-like receptor

A member of the family of evolutionarily conserved receptors that was first described in *Drosophila melanogaster*. These receptors mediate innate immunity and inflammatory responses that can subsequently modulate adaptive immunity in mammals.

Trans-presentation

A process by which the interleukin-15 receptor α -chain (IL-15Rα) presents active IL-15 in *trans* to opposing cells expressing a complex, with a low affinity for IL-15, that contains IL-15Rα and the common cytokine-receptor γ-chain (γ_c), thereby transducing a signal.

Telomere

The segment at the end of chromosome arms, which consists of a series of repeated DNA sequences (TTAGGG in all vertebrates) that regulate chromosomal replication at each cell division.

Telomerase

A ribonucleoprotein enzyme that uses its internal RNA component as a template to synthesize telomeric DNA directly onto the ends of chromosome arms.

Phage display

A technique in which bacteriophages are engineered to express on their cell surface a fusion protein comprised of a foreign peptide or protein and their capsid proteins.

Complementarity-determining region

The hypervariable amino-acid sequences in T-cell-receptor variable regions that interact with complementary amino acids on the peptide–MHC complex.

Myeloablative regimen

Treatment that causes severe bone-marrow suppression requiring administration of haematopoietic stem cells to reconstitute the haematological function of the host and to assure host survival.

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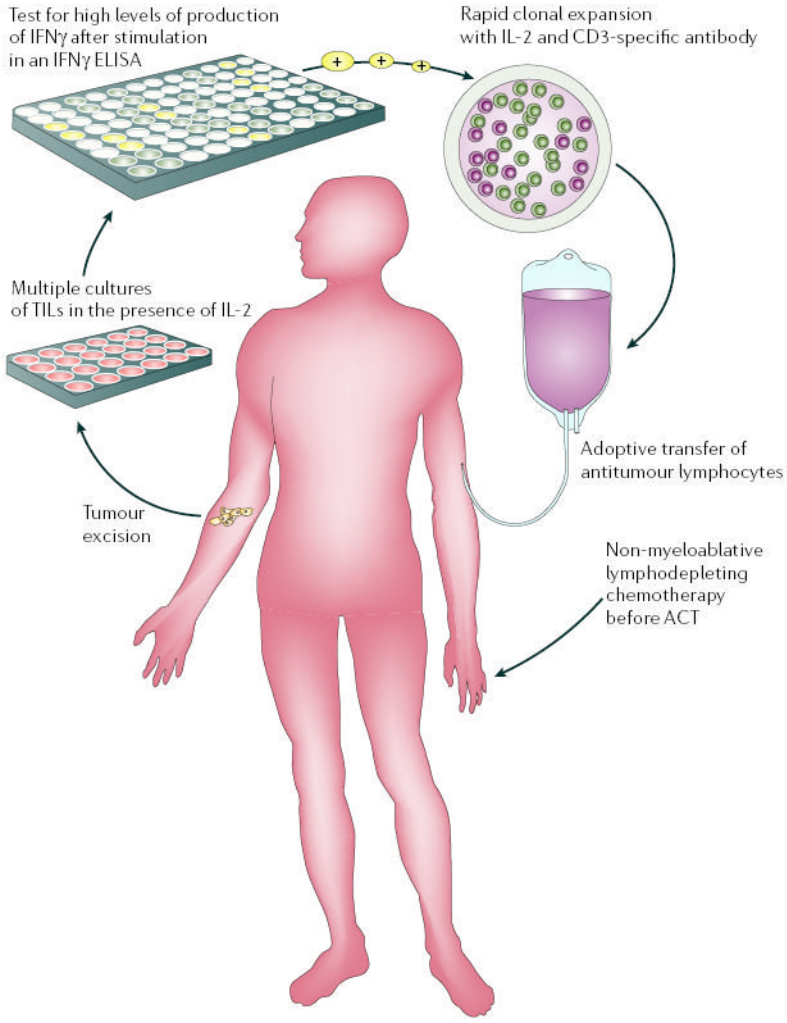

Figure 1. Current clinical protocols for adoptive cell therapy

Adoptive cell therapy (ACT) requires the generation of highly avid tumour-antigen-reactive T cells. Tumour-specific T cells, derived from tumour-infiltrating lymphocytes (TILs), can be efficiently isolated ex vivo from melanoma lesions using high levels of interleukin-2 (IL-2). TILs are successively selected for their ability to secrete high levels of interferon-γ (IFNγ) when cultured with autologous or allogeneic MHC-matched tumour-cell lines. Alternatively, cell-mediated lysis has been used to identify tumour-reactive T cells for transfer. Highly avid, tumour-antigen-reactive T-cell populations selected for ACT are rapidly expanded (to up to 10^{11} cells) using CD3-specific antibody, exogenously supplied IL-2 and irradiated allogeneic peripheral-blood mononuclear 'feeder' cells, and are validated for activity before transfer. Patients now receive systemic immunosuppression before the adoptive transfer of antitumour lymphocytes. Published lymphodepleting regimens consist of a non-myeloablative, but lymphodepleting, conditioning chemotherapy comprised of cyclophosphamide and fludarabine before administration of T cells. Newer, as yet unpublished, regimens also include total body irradiation. ELISA, enzyme-linked immunosorbent assay. This figure is reproduced with permission from REF. [12] © (2005) Elsevier Science.

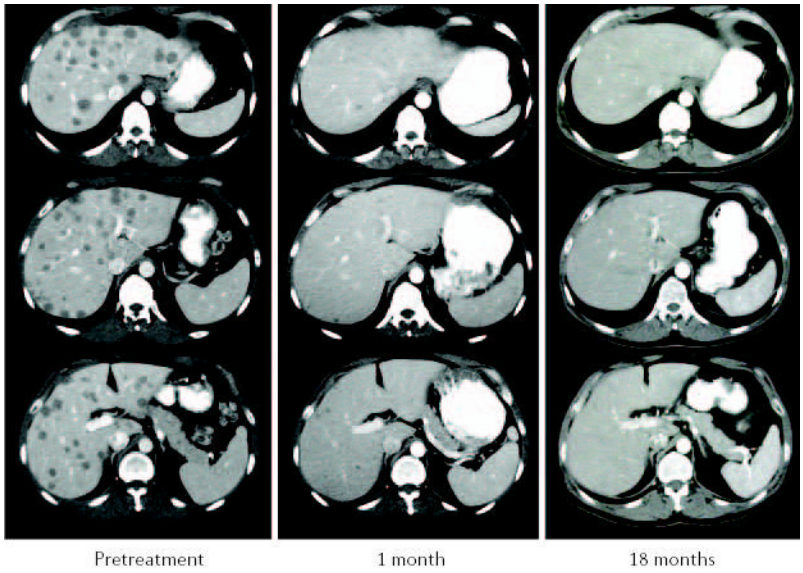

Figure 2. Antitumour response induced by lymphodepletion and adoptive cell therapy

Computed tomography (CT) scans of the liver in a patient with metastatic melanoma show dramatic tumour regression of liver metastases after the administration of tumour-reactive tumour-infiltrating lymphocytes (TILs) following lymphodepletion. The patient is still disease-free after 27 months.

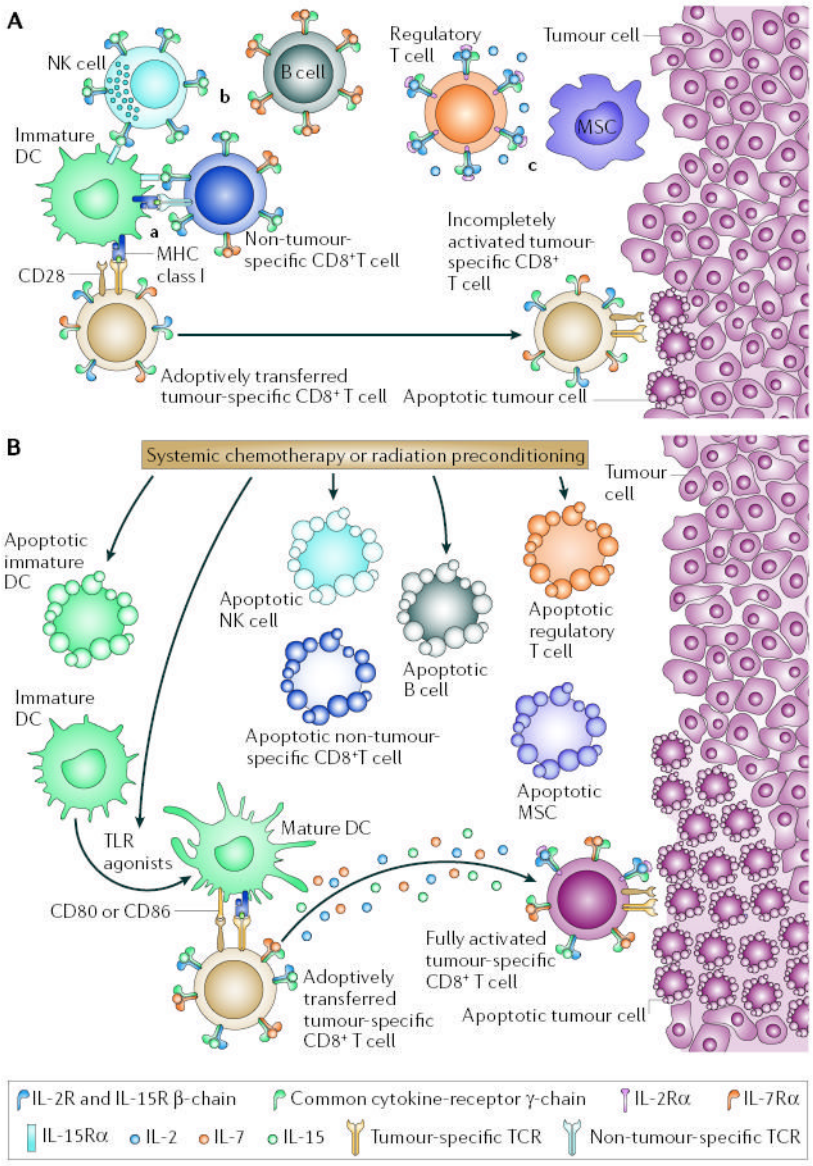

Figure 3. Mechanisms underlying the impact of lymphodepletion on adoptively transferred T cells

A | Adoptive cell therapy (ACT) in a lymphoreplete host. In a lymphoreplete environment, antitumour responses mediated by adoptively transferred tumour-reactive $CD8^+$ T cells might be reduced because of: **a** | competition for antigen at the surface of antigen-presenting cells (APCs) and inefficient lymphocyte activation in the absence of co-stimulatory molecules by immature dendritic cells (DCs); **b** | reduced availability of activating cytokines (including interleukin-2 (IL-2), IL-7 and IL-15) by cellular 'sinks' for these cytokines, which include B cells, T cells and natural killer (NK) cells; and **c** | the suppressive activities of regulatory T (T_{Reg}) cells, myeloid suppressor cells (MSCs) and possibly NK cells. T_{Reg}-cell suppression is mediated by direct T-cell contact and possibly by the release of inhibitory cytokines such as IL-10 and transforming growth factor-β. MSCs mediate T-cell inhibition through direct T-cell contact and the use of enzymes involved in L-arginine metabolism such as the inducible forms of arginase and nitric-oxide synthase, ARG1 and NOS2. **B** | Systemic chemotherapy or radiation before ACT might modify the tumour-bearing host. APCs are reduced in number by direct killing but there might be a net increase in lymphocyte activation because of reduced

competition for antigen at the APC surfaces. At the same time, as a result of the liberation of Toll-like receptor (TLR) agonists after mucosal damage, DCs might be mature, increasing lymphocyte activation. Activating cytokines, such as IL-2, IL-7 and IL-15 might be increased because of the removal of cellular 'sinks'; and T_{Reg} cells, MSCs, NK cells and their suppressive activities are decreased. These modifications might promote the full activation of adoptively transferred tumour-reactive $CD8^+$ T cells and ultimately tumour destruction.

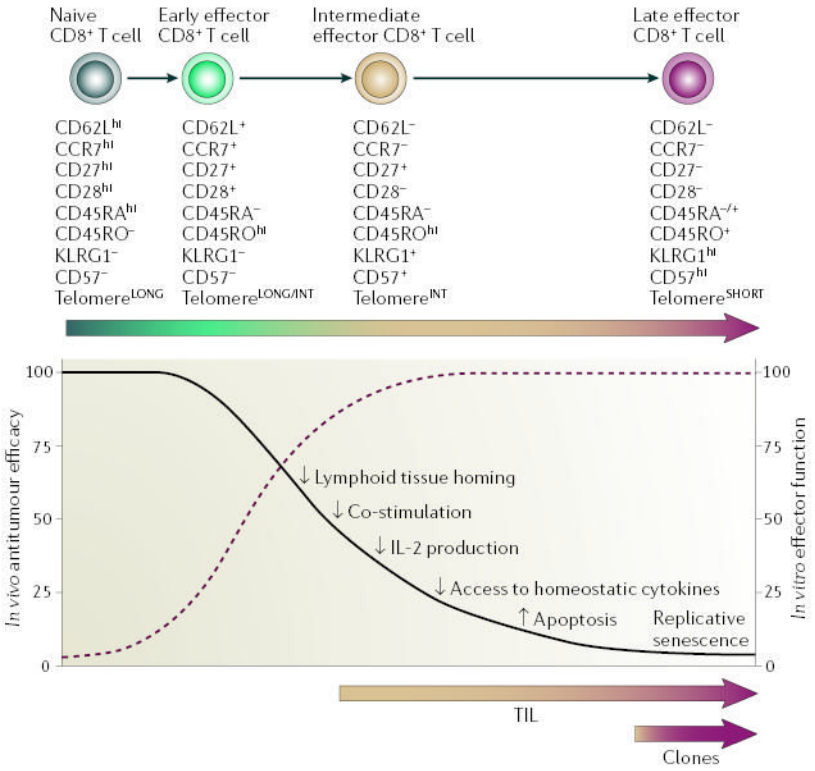

Figure 4. Inverse relationship of *in vitro* and *in vivo* antitumour functions of adoptively transferred naive and effector T-cell subsets

At increasing strength of stimulation, naive $CD8^+$ T cells proliferate and progressively differentiate through early, intermediate and late effector stages. The phenotypic and functional changes that characterize this process are illustrated as no expression (–), intermediate expression (+) and high expression (hi) of the various markers. T cells progressively lose telomere length and proliferative potential, and subsequently become senescent and undergo apoptosis. The progressive acquisition of full effector functions (dashed burgundy line) is associated with a decreased ability of T cells to cause tumour regression after adoptive transfer (black line). The molecular mechanisms underlying this inverse correlation might be comprised of: decreased expression by T cells of lymph-node homing and co-stimulatory molecules, which reduce activation of T cells in vivo; the inability of terminally differentiated T cells to produce interleukin-2 (IL-2); a reduction in the amount of receptors required to receive activating signals from homeostatic cytokines; and finally, an inversion of the expression of pro- and anti-apoptotic molecules with the corresponding acquisition of replicative senescence. Adoptively transferred tumour-infiltrating lymphocytes (TILs) contain several clonotypes with a differentiation state ranging between intermediate and late effector stages, whereas tumour-reactive $CD8^+$ T-cell clones are uniformly late effector T cells. KLRG1, killer-cell lectin-like receptor G1. This figure is reproduced with permission from REF. [65] © (2005) Highwire Press.

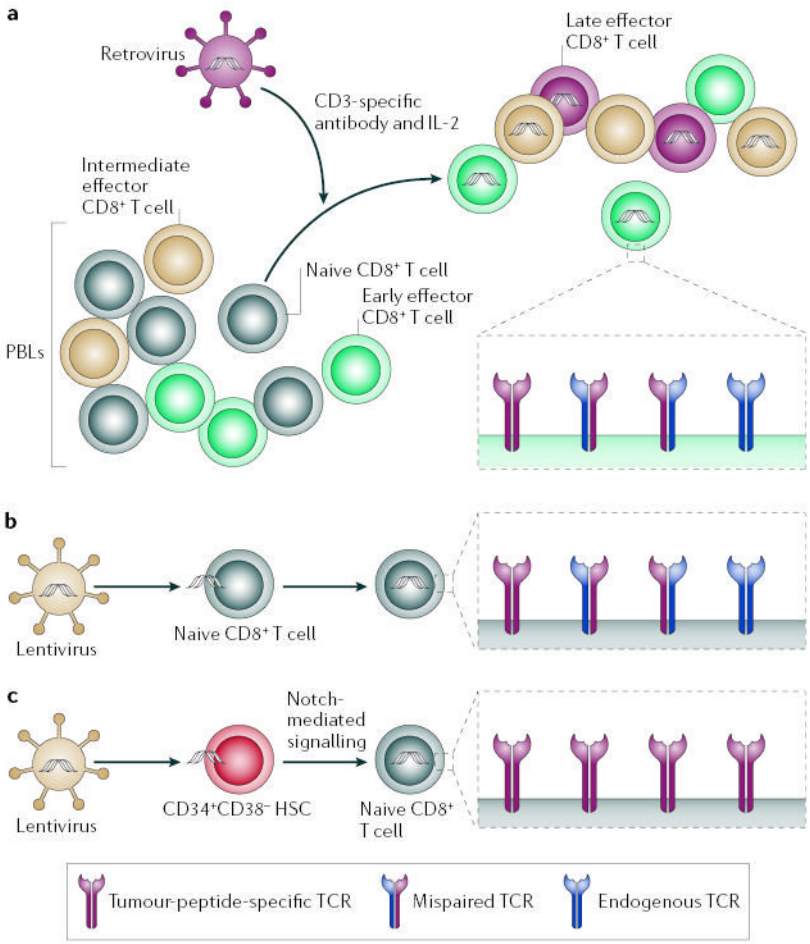

Figure 5. Generation of less-differentiated, central-memory-like tumour-antigen-specific CD8$^+$ T cells by TCR transduction

a | Retroviral transduction of peripheral-blood lymphocytes (PBLs). PBLs at different stages of differentiation, naive (grey), early (green), intermediate (beige) and late effector (burgundy) are activated in vitro with CD3-specific antibody in the presence of interleukin-2 (IL-2) to promote integration of tumour-specific T-cell receptor (TCR) retroviral constructs. This procedure results in the generation of more-differentiated TCR transductants. Pairing with endogenous receptor can reduce the number of tumour-specific TCRs. **b** | Lentiviral transduction of naive CD8$^+$ T cells. Naive CD8$^+$ T cells isolated through selective sorting can be transduced with tumour-specific TCR by using lentiviral constructs that do not require activation and consequent differentiation. Pairing with endogenous receptor can reduce the number of tumour-specific TCRs. **c** | Lentiviral transduction of haematopoietic stem cells (HSCs). CD34$^+$CD38$^-$ HSCs isolated though selective sorting can be transduced with tumour-specific TCR using lentiviral constructs. HSCs can be induced to differentiate into naive CD8$^+$ T cells in vitro through Notch-mediated signalling. Repression of recombination-activating genes by the transduced tumour-specific TCR allows for the uniform expression of tumour-specific TCRs.