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Engineering lymphocyte subsets: tools, trials and tribulations

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Abstract

Cell-based therapies with various lymphocyte subsets hold promise for the treatment of several diseases, including cancer and disease resulting from inflammation and infection. The ability to genetically engineer lymphocyte subsets has the potential to improve the natural immune response and correct impaired immunity. In this Review we focus on the lymphocyte subsets that have been modified genetically or by other means for therapeutic benefit, on the technologies used to engineer lymphocytes and on the latest progress and hurdles in translating these technologies to the clinic.

The adoptive transfer of various mature lymphocyte subsets into patients with the goal of treating a disease or correcting a congenital immunodeficiency is an old concept that has recently gained momentum in the clinic. Both allogeneic and autologous lymphocytes have been used over the years. Perhaps the most potent therapeutic benefit yet realized with unmodified lymphocytes is the ‘allogeneic effect’ — the tumoricidal activity that follows an infusion of allogeneic lymphocytes. It was appreciated only retrospectively that the powerful and durable antitumour effects of bone marrow transplantation can largely be ascribed to allogeneic T cell transfer. The interest in genetically modifying lymphocytes has increased dramatically in recent years, as several basic and translational scientists have concluded that the modification of autologous lymphocytes should enable the creation of pharmacologically enhanced immune cells that are more potent and have a larger therapeutic window than allogeneic T cell transfer[1]. In 1990, the first study using genetically modified T cell infusions in patients with cancer was reported. In this study, retrovirus-mediated insertion of the neomycin phosphotransferase gene into the genome of lymphocytes was used to track tumour infiltration following infusion[2]. The goal of these studies was to mark but not pharmacologically alter the function of the infused T cells. The study was a clinical success in that there was no significant toxicity; however, scientifically there was much room for improvement as at 1 week after the infusion barely 0.01% of the transferred cells remained in the circulation. Significant progress has been achieved since then, and in this Review we describe the increasing range of tools that are available to modify lymphocytes, the various lymphoid subsets and lymphoid progenitors that are suitable for use in immunotherapies, and the potential safety issues and clinical progress in the fields of immunotherapy for autoimmunity, cancer and infectious diseases.

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Choosing the tools

Advances in basic science have presented numerous approaches to engineer lymphocytes at the genomic, RNA, epigenetic and protein levels, with the goal of pharmacologically enhancing the immune system.

Virus vector-based approaches

Recombinant adenoviruses and adeno-associated viruses have been the main vectors used for human gene transfer research that involves targeting cells that are not derived from haematopoietic cells. However, although adeno-associated viruses can mediate site-selective integration into the target cell genome under some conditions, it has not been successfully applied to T cells. An overview of viral vectors proposed for engineering the immune system is shown in Table 1. For lymphocyte-based therapies, chromosome-integrating vectors that are derived from gammaretroviruses or lentiviruses have been most useful for long-term gene expression because of their ability to integrate into the host genome, a feature that can result in permanent expression of the transgene, and for their low intrinsic immunogenicity[3]. The use of gammaretroviruses is somewhat cumbersome because it requires the induction of cell replication for vector integration and, as discussed below, there may be more safety concerns associated with gammaretroviruses than with lentiviruses. Lentivirus-derived vectors are more efficient for gene transfer, in part because of their ability to integrate into the genome of non-dividing cells[4,5] and because, in some circumstances, they are less susceptible to gene silencing by host restriction factors[6,7].

Foamy virus vectors are derived from the Spumavirus genus of retroviruses[8] and may have advantages over gammaretroviruses and lentiviruses, of which the most important is that the wild-type foamy virus seems to be non-pathogenic in non-human primates and humans. These vectors will soon enter clinical testing and have promising integration properties that may prove to render them the safest of the integrating viral vectors[9–11].

In contrast to recombinant viral vectors derived from human adenovirus serotype 5 (Ad5), viral vectors derived from both Ad5 and Ad35 (Ad5–35 vectors) were reported to mediate gene transfer in up to 10% of resting T cells and 30–45% of T cells after activation with phyto-haemagglutinin[12]. we found that Ad5–35 vectors resulted in gene transfer in more than 90% of T cells after activation by CD3- and CD28-specific antibodies[13]. Ad5–35 vectors have promise for adoptive transfer of engineered lymphocytes in clinical situations where expression of a transgene for less than a week is required. In contrast to retroviral vector-transduced cells, the Ad5-35 vector transgene will not persist because adenovirus-based vectors do not integrate into the host genome. It is not yet known if residual adenovirus proteins will render the Ad5-35-transduced T cells immunogenic and/or impair their persistence.

Non-virus-based approaches

The use of approaches to engineer lymphocytes that do not involve viral vectors has increasing promise (Table 1). electroporation can now efficiently introduce plasmid DNA into lymphocytes for transient expression of transgenes[14]. However, electroporation can occasionally result in genome integration, although this occurs at much lower frequencies than with virus-based approaches, resulting in stable expression and the accompanying potential for genotoxicity The first clinical trial testing the adoptive transfer of T cells engineered using electroporation was recently reported[15], and although this approach was safe, the cells were short-lived after transfer, probably owing to the long-term culture of the cells that was necessary to achieve sufficient integration efficiency.

Transposon-based systems can integrate transgenes much more efficiently than plasmids that do not contain an integrating element[16,17]. Transposons are most commonly introduced into cells by electroporation or lipofection and they contain nuclear localization signals that, in the presence of transposase enzymes, integrate into the genome at 5′-TA-3′ sequences. various transposase-based systems are now entering clinical trials to test the safety and feasibility of this approach to engineer T cells[18].

Non-viral vectors have several advantages over viral vectors in engineering cells, including lower financial costs and perceived safety benefits. clinical-grade plasmid DNA-based approaches are substantially less expensive than approaches using recombinant viral vectors. what is less certain is the safety profile of these approaches. Gammaretroviruses and lentiviruses have a proven safety record for use in human T cell engineering[19,20], whereas the relative genotoxicity of transposons is unknown. The integration sites of lentivirus-based vectors are not random and do not seem to favour proto-oncogenes[21]. It remains unknown whether transposon-based systems will be more or less favourable in this regard and whether they can reach the efficiencies that are achieved by virus-based integration. Approaches to achieve site-specific integration and DNA editing are described in Box 1. These approaches are just entering clinical trials and, if found to have sufficient efficiency, should allay concerns regarding lymphocyte engineering fusing non-site-specific integration approaches.

RNA engineering

For some applications, genomic alteration of adoptively transferred lymphocytes may not be required to achieve substantial therapeutic effects (Box 2). RNA-based electroporation of lymphocytes using *in vitro* transcribed mRNA mediates transient expression of proteins for approximately 1 week, and redirected T cells transduced with RNA encoding T cell receptors (TCRs) or chimeric antigen receptors have the expected gains of function[22,23]. This approach is attractive because RNA transduction efficiencies can approach 100% and the immediate toxicity is much lower than that of approaches using electroporation of plasmid DNA. other investigators have successfully modified T cell functions using RNA transduction of chemokine receptors and cytokines[24,25]. Furthermore, RNA-based approaches are expected to be less genotoxic than approaches that intentionally modify the genome. Transposase enzymes can be delivered as mRNA[26], thereby avoiding the possibility of genomic integration and continued chromosome ‘hopping’ (REF. 27).

In addition to mRNA engineering, various approaches using regulated expression of microRNAs might be used for pharmacological alteration of lymphocyte function, as first shown for microRNA-181 (REF. 28). The role of microRNAs in the regulation of lymphocyte function is reviewed in REF. 29. clinical trials using mRNA-transduced dendritic cells have been safely conducted[30] and trials using mRNA-electroporated lymphocytes are being initiated by several groups.

Epigenetic engineering

In addition to the expression of master transcription genes[31], recent studies have shown that several epigenetic events, including DNA methylation, histone modifications and changes in chromatin structure, can affect lymphocyte subset differentiation and function[32]. Epigenetic marks can be heritable but are inherently plastic because the DNA sequence remains unchanged, and in theory these marks provide an approach for epigenetically modified adoptive cell therapy, which is expected to be less durable than the use of genetically engineered cells but intrinsically safer. In mammalian cells, ~5% of the deoxycytidine residues in DNA are present as 5-methyl-deoxycytidine residues[33], which are primarily found in CpG motifs. methylation occurs immediately after DNA replication and involves the transfer of a methyl group in a reaction catalysed by DNA methyl-transferases

(DNMTs). In general, methylation of DNA represses gene expression, whereas demethylation results in gene activation[34]. In early studies, the inhibition of DNA methylation with the incorporation of azacytidine in cell culture was shown to promote interleukin-2 (IL-2) secretion in transformed cell lines[35], and recent studies indicate that epigenetic regulation of the regulatory T (T_{Reg}) cell-associated transcription factor forkhead box P3 (FOXP3) can be predictably controlled with dNmT inhibitors to generate functional, specific and seemingly stable T_{Reg} cells[36]. However, the use of DNMT inhibitors *in vivo* can also augment the effects of adoptively transferred conventional T cells[37]. The demethylation agent 5-aza-2′-deoxycytidine, decitabine (dacogen; eisai), has been approved by the uS Food and drug Administration for the treatment of patients with myelodysplastic syndromes and may be useful for various adoptive transfer strategies.

Histone deacetylase (HDAC) inhibitors have been shown to be potent inducers of immunosuppressive pathways. T_{Reg} cells express more HDAC9 than do conventional T cells, and systemic treatment with HDAC inhibitors stimulates T_{Reg} cells[38], as does the incorporation of various HDAC inhibitors during *in vitro* cell culture (T. Akimova and w. w. Hancock, personal communication). However, systemic therapy with less specific HDAC inhibitors also mediates direct antitumour effects[39] and augments the antitumour effects of adoptively transferred conventional T cells[40]. Thus, the incorporation of various DNMT inhibitors and HDAC inhibitors in cell cultures as an approach to engineer lymphocyte subsets has the potential to augment T cell function and maintain fidelity *in vivo*. Indeed, it is conceivable that the effects of inhibitors on lymphocyte function during *in vitro* engineering may be more pronounced and selective because *in vivo* therapies with these inhibitors can have opposing effects owing to indirect effects on the tumour, the tumour microenvironment or antigen presentation that are distinct from the direct effects on lymphocyte subsets. Further studies are required to clarify the pleiotropic and tissue-specific expression of DNMT and HDAC inhibitors[41] in order to guide their use for optimally augmenting or suppressing lymphocyte functions. However, it is already clear that the intentional epigenetic remodelling of lymphocytes has the potential to enhance or suppress lymphocyte effector functions.

Protein transduction

Advances in fusion protein technology allow high transduction efficiencies for many mammalian cell types. Proteins exceeding 100 kDa can be introduced into cells by incorporation of small protein transduction domains (PTDs) derived from the HIV protein Tat, the antennapedia homeodomain from *Drosophila melanogaster* or the VP22 protein from herpes simplex virus (HSV)[42]. Recent advances of the technology allow the delivery of packaged synthetic small interfering RNA (siRNA) duplexes into cells[43]. The addition of recombinant proteins and siRNA complexes to cell culture approaches has several potential uses for engineering lymphocytes. First, the HIV Tat PTD has been shown to efficiently introduce peptides into the mHc class I pathway for presentation by antigen-presenting cells (APCs), and the antigen-loaded APCs then efficiently prime cytotoxic T lymphocyte (CTL) responses[44]. Because human T cells, unlike mouse T cells, can efficiently function as APCs to prime CTL responses[45,46], protein transduction technology could be used to load T cells *in vitro* with desired antigens for an adoptive transfer-based vaccine approach. Because proteins fused to PTDs can pass through cell membranes and retain biological function, they also have the potential to modify lymphocyte function in a cell-autonomous manner. In recent studies, the cytoplasmic domain of cytotoxic T lymphocyte antigen 4 (CTLA4) fused to a PTd has been introduced into T cells and shown to specifically inhibit TCR signalling[47], and the systemic administration of CTLA4-PTD ameliorated collagen-induced arthritis in mice[48]. Recent studies indicate that bispecific antibodies targeting CD3 and CD19 can mediate substantial antitumour effects that are presumably MHC independent in patients

with lymphoma[49], and thus the use of protein-based transduction and a related strategy, known as protein painting[50], has significant opportunity to engineer enhanced lymphocyte functions without the risks of gene transfer approaches.

General considerations

The ultimate choice of the technology used to engineer cellular immunity should be based on several scientific and practical considerations. Scientific considerations include the size of the transgene, the number of transgenes, the requirement for permanent or transient genetic modification, the transgene expression level (high or low) and whether constitutive or inducible transgene expression is desired. For therapy with engineered lymphocytes using integrating vectors, a key issue facing the field is whether the risk of oncogenic transformation is greater with retroviral vectors than with lentiviral vectors. None of the clinical trials carried out so far using T cells genetically modified by gammaretrovirus vectors has reported adverse events due to insertional mutagenesis. cyclin-dependent kinase inhibitor 2A (CDKN2A) is a tumour suppressor protein and, in tumour-prone mice engrafted with CDKN2A-deficient bone marrow, a gammaretrovirus vector was found to accelerate tumorigenesis, whereas a lentivirus vector, present at higher copy number, did not[51,52]. For approaches that require integrated transgenes, targeted integration is preferable to the integration patterns of retroviruses. However, at present, only zinc-finger nucleases (ZFNs) can mediate targeted integration, and the safety and feasibility of this approach is still unknown (Box 1). Some strains of adeno-associated virus can mediate targeted integration; however, to date this has not been possible with T cells.

Practical considerations that guide the type of cellular engineering used include the cost of products for cell culture and the availability of clinical-grade vector systems. These considerations should be based on the target product profile of the engineered lymphocytes. In some instances only a brief persistence of adoptively transferred lymphocytes may be required, whereas in others lifelong persistence seems desirable. For example, therapy with engineered lymphocytes is ongoing for patients with advanced cancer[53] and persistant therapy has been proposed for patients with type 1 diabetes[54] or multiple sclerosis[55]. ethical considerations would dictate that a much higher level of safety would be required for the engineered lymphocytes used for type 1 diabetes, a chronic disease for which life-sustaining but non-curative therapy is available, than those used for patients with cancer who do not have long-term therapeutic options.

Choosing the delivery service

Mature T cells are among the most suitable cells for modification, and stable modification has been achieved using several approaches. The efficiency of lymphocyte modification has been consistently higher than with haematopoietic stem cells (HSCs)[19], such that with current technologies transgene delivery and expression in T cells is not limiting. An overview of cell culture approaches used for various lymphocyte subsets that have been tested in human trials is shown in Fig. 1.

Conventional T cell subsets

Immunotherapy with engineered T cells is attractive for several reasons, including the long-lived persistence that has been shown in humans following adoptive transfer[56]. A major advantage of adoptive cell therapy is that the therapeutic effects can be augmented by isolating the lymphocytes that have desired effector or regulatory properties while removing the cells that may have antagonistic effects. clinical studies with effector T cells are the most advanced, and have progressed to a Phase III clinical trial testing the efficacy of T cells that are transduced to express an HSV thymidine kinase (HSV-TK) conditional ‘safety switch’ in

the setting of haploidentical stem cell transplantation for high-risk acute leukaemia in remission[57]. In this approach, allogeneic T cells can mediate antitumour effects in the context of allogeneic stem cell transplantation, and in the event of significant graft-versus-host disease (GVHD) the cells can be ablated by administration of the antiviral drug ganciclovir; the cells expressing HSV-TK are selectively killed because HSV-TK mono-phosphorylates ganciclovir (a guanosine analogue), which is eventually converted to the toxic triphosphate form and incorporated into replicating DNA where it causes chain termination and cell death.

At present, there is considerable enthusiasm for the approaches involving the adoptive transfer of engineered $CD8^+$ CTLs. The transfer of MHC class I-restricted TCRs can 'convert' a population of polyclonal $CD8^+$ T cells to CTLs of monoclonal TCR specificity[58]. This approach is attractive because high-affinity CTLs of appropriate specificity are generally lacking in patients with advanced cancer or chronic infections. Thus, the introduction of TCRs with high affinity or even a supraphysiological affinity has the potential to increase the recognition and killing of tumour cells that have low expression of cognate peptide-MHC class I complexes. In the case of HIV infection, high-affinity TCRs seem to have an improved ability to control viral infection and to delay the appearance of virus escape mutants[59]. clinical trials testing the adoptive transfer of engineered TCRs for cancer have been reported with promising clinical results and serious but generally reversible tissue-specific 'on target' toxicity[60].

Since being first reported in mice more than two decades ago[61], the transfer of $CD8^+$ T cells engineered to express MHC-unrestricted chimeric antigen receptors is now rapidly advancing in human trials. chimeric antigen receptors have the potential to serve as an 'off the shelf ' reagent to redirect T cells with cytotoxic or regulatory functions to desired cell surface ligands of various tumour, stromal and viral targets[62]. Recently, the first report to show clinical antitumour effects with chimeric antigen receptors investigated the effects of adoptively transferred autologous T cells, expressing a chimeric antigen receptor that targeted the diasialoganglioside GD2, in patients with advanced neuroblastoma[63]. This trial used a first-generation chimeric antigen receptor comprised of a TCRζ domain, and improved methods of cell culture were shown to contribute to enhanced persistence of the adoptively transferred chimeric antigen receptor modified T cells. In particular, the subset of T cells with antigen-specific chimeric antigen receptors specific for epstein-Barr virus had improved *in vivo* survival, probably because they received appropriate co-stimulation from professional APCs expressing epstein-Barr virus antigens. more advanced chimeric receptor and vector designs incorporating multiple co-stimulatory domains and lentiviral vector technology are now entering clinical trials at several centres[64,65].

The adoptive transfer of engineered $CD4^+$ T cells has promise for adoptive therapy of cancer and HIV (reviewed in REFS 19,66). A recent intriguing study found that *in vitro*-polarized T helper 17 (T_H17) cells were more effective in mediating regression of B16 melanoma than unpolarized T_H0 cells or T_H1 cells[67]. The adoptive transfer of T_{Reg} cells has the potential to prevent GvHd and solid organ transplant rejection and to prevent or treat autoimmunity[68]. engineered T_{Reg} cells have been proposed for the treatment of autoimmune disease based on results from preclinical models. In one model, genetic modification of polyclonal T_{Reg} cells with a chimeric antigen receptor consisting of a myelin basic protein epitope bound to the extracellular and transmembrane domains of an mHc class II molecule linked to the cytoplasmic domain of the TCRζ chain resulted in functional T_{Reg} cell activation following recognition of these modified T_{Reg} cells by myelin basic protein-specific T cells, thus preventing or reducing the lethality of experimental autoimmune encephalomyelitis[69]. others have shown that T_{Reg} cells engineered to express chimeric antigen receptors have potential for the treatment of colitis[70]. one issue with redirected T cells has been whether sufficient

survival of T cells expressing chimeric antigen receptors will occur to mediate long-term effects. It has been shown that endogenous cytotoxic $CD4^+$ T cells specific for varicella zoster virus (VZV) can be engineered to express tumour-specific chimeric antigen receptors. The VSV-specific T cells can be expanded *in vivo* by stimulation of their native TCR by administration of VZV vaccine, but they retain the ability to recognize and lyse tumour targets in an MHC-independent manner through the VZV-specific chimeric antigen receptor[71]. Together, these results are important because target cells in patients with cancer and chronic viral infections often have decreased expression of antigen-loaded MHC molecules.

Studies indicate that, on a per-cell basis, the adoptive transfer of T cells with extensive replicative capacity have improved engraftment and antitumour effects than transfer of terminally differentiated effector T cells that have a more potent cytotoxic effector function[72]. This might be because central memory (T_{CM}) T cells can self renew and differentiate into effector T cells *in vivo*, whereas terminal effector memory T (T_{EM}) cells have lost this plasticity[73,74]. A controversy has arisen in the field as to how best to generate T cells with good replicative capacity and the desired function. One approach is to isolate T_{CM} cells with the desired specificity *in vitro* by sorting or other means of physical separation, engineer the desired antigen specificity, expand and then infuse the T_{CM} cells[75]. we have proposed that manipulation of bulk T cell culture conditions can enrich and maintain T_{CM} cells, and thereby remove the need for cell sorting procedures. our group and others have found that cell culture conditions that augment co-stimulation through CD28 and 4-1BB (also known as CD137) promote the maintenance of T_{CM} cells *in vitro*[76-78]and *in vivo*[79-81]. The use of memory stem cells (that is, T cells programmed for the most extensive self renewal) has significant potential[82].

γδ T cells

Recent advances in our understanding of the biology of γδ T cells suggest that these cells have promise as a molecularly targeted immunotherapy, with several properties that αβ T cells lack. There is evidence that both αβ T cells and γδ T cells have a role in tumour immunosurveillance[83,84], although the relevance of γδ T cells to tumour immunosurveillance for certain tumours has been questioned[85]. Human γδ T cells can kill various primary carcinomas[86], and a recent study indicates that they are cytotoxic to cancer-initiating cells[87]. Another feature of human γδ T cells is that they can prime αβ T cells with an efficiency similar to that of dendritic cells[88], and thus adoptive transfer of γδ T cells could serve to augment cytotoxic T cell effector functions. unlike αβ T cells, γδ T cells are activated by various non-peptide endogenous ligands that are expressed on stressed cells. clinical grade synthetic ligands are now available that allow the efficient expansion of the major subset of $V\gamma9V\delta2^+$ T cells in peripheral blood, or cells may be treated with aminobisphosphonates such as zoledronate (Zometa/Aclasta/Reclast; Novartis)[89], which are agents that increase the expression of endogenous ligands such as isopentenyl pyrophosphate, a steroid intermediate that promotes the expansion of γδ T cells (see Supplementary information S1, (figure)). Human γδ T cells in the blood seem to be more terminally differentiated, whereas those in tissues are less differentiated. It is not known how the population of $v\gamma9v\delta2^+$ T cells in the blood are maintained, as at present they do not have an extensive proliferative capacity *in vitro*, unlike αβ T cells[86]. It is possible that optimal conditions for *in vitro* propagation have not yet been identified and, consistent with this, a recent report showed that inclusion of IL-2 and IL-21 in the culture medium with the synthetic ligand isopentenyl pyrophosphate generated γδ T cells with more potent cytotoxicity and higher T_H1-type cytokine secretion than if only IL-2 is used[90].

Adoptively transferred γδ T cells can eradicate human tumour xenografts in various SCID mouse models[91], and two recent clinical studies have shown the safety and feasibility of

adoptive transfer of peripheral blood Vγ9Vδ2$^+$ T cells stimulated with the synthetic ligand 2-methyl-3-butenyl-1-pyrophosphate and IL-2 (REF. 92) or isopentenyl pyrophosphate and IL-2 (REF. 93).

Human γδ T cells can be engineered to express non-MHC-restricted chimeric antigen receptors[94] and αβ TCRs[95]. The expression of additional TCR chains in T cells can lead to the generation of T cells with unknown specificity owing to the formation of mixed dimers between the endogenous and introduced TCR chains[96]. The use of γδ T cells may provide a significant safety feature over the use of TCR-ENGINEERED αβ T cells, taking advantage of the finding that α and β TCR chains cannot pair with γ and δ TCR CHAINS. Nevertheless, it remains unclear whether the adoptively transferred γδ T cells will home to tumours or sites of inflammation and, if so, what is the best source of γδ T cells. Blood-derived vγ9vδ2$^+$ T cells are the most convenient cells to harvest and have cytotoxic effector functions against various tumours[86], but vγ9vδ2$^-$ T cells derived from tissues may be preferable as they may have longer persistence and improved homing capacity, especially for patients with tumours located in the skin and intestines, sites that are preferentially targeted by γδ T cells.

Natural killer T cells

Natural killer T (NKT) cells are functionally related to γδ T cells in that they also bridge innate and adaptive immune responses and can enhance or suppress immunity[97]. The best characterized human NKT cell subpopulation, referred to as invariant NKT (iNKT) cells, expresses CD161 and an invariant Vα24Jα18 TCR that recognizes α-galactosylceramide (α-GalCer) presented by the MHC class I-like molecule CD1d. After activation, iNKT cells have MHC-independent cytotoxic activity for various tumours and secrete high levels of interferon-γ (IFNγ), although this function becomes impaired in patients with cancer[98]. compared with mouse NKT cells, human NKT cells are rare (<1% of total lymphocytes). However, human NKT cells, unlike mouse NKT cells, can undergo substantial expansion *in vitro* after repeated stimulation with α-Galcer-pulsed APCs, similar to conventional αβ T cells stimulated with peptide-loaded APCs[99]. The first adoptive transfer studies of iNKT cells have been conducted by adapting this method to a clinical-scale process. expanded iNKT cells were administered intravenously to patients with lung cancer[100] and by intra-arterial injection to patients with squamous cell carcinoma of the head and neck[101]. The results were promising, demonstrating safety, feasibility and some evidence of antitumour efficacy. In mice, iNKT cells have also been shown to have immunosuppressive effects[97], so caution must be taken for their use in patients with cancer, but this may mean that they have potential for the treatment of autoimmunity.

Future considerations for engineered lymphocyte therapy

Robust clinical-scale culture technologies have now been developed for αβ T cells, T_{Reg} cells, γδ T cells and iNKT cells, allowing for the first time adoptive transfer approaches with diverse subsets of engineered lymphocytes. For clinical scenarios in which long-term engraftment of the adoptively transferred T cells is desired αβ T cells are preferable, and the extensive proliferative capacity that iNKT cells display *in vitro* indicates that they may have similar enhanced persistence. However, for some applications, short-term engraftment provided by γδ T cells may be sufficient or even desired for situations in which long-term engraftment might cause toxicity. In this case, short-term persistence of the engineered cells would replace the need to include conditional safety switches. Another attractive feature of γδ T cells is the possibility of expressing αβ TCRS in these cells without the risk of generating mixed heterodimeric TCRs. In addition, the distinct homing properties and intrinsic high level cytotoxicity of engineered γδ T cells and iNKT cells together may have additional or even synergistic therapeutic effects over the use of only αβ T cells. Finally,

various approaches using lymphocytes as vehicles to deliver cargo to tumours such as oncolytic viral vectors have been proposed[102], and redirected γδ T cells and iNKT cells may be preferred for these approaches to increase the delivery of oncolytic vectors and take advantage of the ability of these cellular carriers to protect from systemic viral neutralization.

when our understanding of lymphocyte development improves, another area of opportunity will be the adoptive transfer of engineered precursor cells that are programmed to have the functions of effector lymphocytes. examples include the adoptive transfer of engineered common lymphoid progenitor cells[103], memory stem cells[82] and 'retrogenic T cells', which are embryonic stem cells or HSCs engineered to express transgenic TCRs or chimeric antigen receptors after they differentiate into T cells *in vivo*[104–107].

Genotoxicity and other safety considerations

Lymphocyte engineering procedures can take place *in vitro* or can be done *in vivo* using various targeting strategies[108-110]. To date, only strategies using *in vitro* engineering and integrating vectors have reached clinical trials, in part owing to the inherent safety of *in vitro* approaches. A general feature of gene transfer strategies using *in vitro* engineered lymphocytes is that the potential toxicity is diminished and more predictable than after *in vivo* gene transfer approaches.

There are several potential safety concerns with engineered lymphocytes. The safety profile of autologous non-modified lymphocyte adoptive transfers is well established (reviewed in REF. 111) and the only common adverse events have been attributed to cytokine release, which is well tolerated with premedication, and manifestations of autoimmunity such as vitiligo and thyroiditis. Recently, we have observed colitis and skin rashes resembling GVHD in patients given non-modified autologous T cells under conditions that promote homeostatic expansion of the transferred cells[112]. In addition to these adverse effects of unmodified lymphocytes, the toxicity due to transfer of engineered T cells is expected to include various 'on-target' and 'off-target' toxicities from transgenes, as well as vector-specific toxicity.

Transgene-specific toxicity

Toxicity attributable to the specificity of the transgene has been observed and for future trials the expected toxicities should be considered on a case by case basis, depending on the particular modification. on-target toxicity can occur as a consequence of the modified lymphocyte reacting to the structure targeted. This was reported in the first trial of gene-modified T cells expressing an HLA-A2-restricted TCR specific for MART1 (melanoma antigen recognized by autologous T cells 1; also known as MLANA) antigen[113]: both antitumour effects (on-target efficacy) and toxicity at non-tumour sites that also express MART1, such as the skin, ears and eyes, (on-target, off-organ toxicity) were observed. The most extreme experience with on-target, off-organ toxicity occurred in a clinical trial in which patients were given T cells engineered to express a chimeric antigen receptor that was specific for carbonic anhydrase IX (CAIX), a transmembrane protein that is overexpressed by cancerous kidney cells. In three of three patients, severe liver toxicity ensued within 1 week following infusion of the gene-modified T cells[114,115]. Subsequent investigation revealed that the CAIX protein was also expressed in the biliary tract of the liver, illustrating the need to carefully pick the target as well as the potent effects of engineered lymphocytes.

For ethical and both scientific and lay perception issues, genetic engineering is held to a higher safety standard than many other experimental therapies. To date, there is promising efficacy following the infusion of gene-modified T cells in various clinical trials, and no

serious adverse events that are a result of the engineering process have been reported. mature lymphocytes are intrinsically resistant to transformation; however, the specific context of the genetic engineering can increase the risk of transformation. For example, a serious concern has recently been reported following the introduction of a vector that encoded IL-15 into human CTLs[116]. The cells exhibited logarithmic *in vitro* growth in the absence of exogenous cytokine support for more than 1 year after transduction with a gammaretrovirus-based vector encoding IL-15. The clone exhibited constitutive telomerase activity, and the presence of an autocrine loop was suggested by impaired cell proliferation following knockdown of IL-15 receptor α-subunit expression. The authors concluded that although the cells have the theoretical promise to generate T cells with enhanced effector qualities, this particular approach would have unacceptable risk for genotoxicity in the context of a clinical application. However, the beneficial effects of cytokine gene transfer in T cells can be substantial and the addition of a conditional suicide gene has the potential to ensure the safety of the approach.

The increasing interest in the use of lymphocytes engineered to express transgenic TCRs has raised concerns for new forms of off-target toxicities that may occur[117]. For example, the transgenic TCRs may exhibit imperfect allelic exclusion and mispairing can occur with the endogenous and transgene TCR chains, leading to the generation of TCRs with new specificities that have not been selected for tolerance in the thymus. Toxicity from ‘mixed TCR dimers’ has not yet occurred in clinical trials; however, it is premature to conclude that this will not occur, as there is at present insufficient clinical experience with T cells expressing transduced TCRs. Finally, using phage display technology to engineer TCRs and lentiviral vectors[118], our group has recently reported that T cells engineered to express TCRs with enhanced affinity to their cognate antigen have greater antiviral activity than the parental TCR-modified T cells[59]. It is possible that picomolar affinity variants of TCRs may have off-target specificities through the development of crossreactivity to other mHc proteins or degenerate peptide recognition.

Vector-specific toxicity

Lymphocytes modified by genome-integrating vectors can be expected to have various toxicities (Box 3). The most serious effect reported to date is cell transformation. It is well documented that gammaretroviruses can cause insertional activation of proto-oncogenes in animals, resulting in cancer[119]. As the human genome comprises approximately 3×10^9 bases, if transgene integration into cellular alleles with an average target size of 10 kb is a random process, then integrations into each gene would be expected to occur at a frequency of 1 in 300,000 integrations[120]. As 10^7 to 10^9 lymphocytes are typically transduced in current clinical trials, each gene would be expected to be disrupted or mutated often, with the most frequent outcome being the generation of a null allele. Recent studies indicate that the mouse mammary tumour virus, a gammaret-rovirus, has a nearly random dispersion of integration sites[121]. Gammaretroviruses integrate preferentially near transcription start sites, and may be particularly prone to cause insertional activation of proto-oncogenes. By contrast, lentiviruses preferentially target transcription units, and analysis of the integration-site positions in the human genome revealed that the HIV-1 integration sites are clustered[122]. Initial characterization of the integration sites in patients with HIV or AIDS receiving adoptively transferred lentivirus-engineered $CD4^+$ T cells indicates that the pattern is similar to that of wild-type HIV-1 integration sites[20]. In particular, no integrations were identified within 50 kb of proto-oncogenes or known tumour suppressor genes. These empirical data, in conjunction with natural history data from patients with HIV or AIDS who have not developed T cell leukaemia, are consistent with a favourable profile for genotoxicity of lentivirus-engineered lymphocytes used in clinical trials. whether this will

prove to be true for lentivirus-engineered HSCs and progenitor cells remains to be established.

Are engineered T cells 'safer' than engineered HSCs?

The development of retroviral vectors provided the first means to engineer lymphocytes at efficiencies that would support their use in clinical trials. of historical interest, bone marrow-derived HSCs were proposed for the first clinical trials involving gene transfer therapy commencing with the development of efficient gamma retrovirus-based vectors in the early 1980s[123]. However, the promise of engineered HSCs that could generate a permanently engineered immune system — an immunological 'holy grail' — has not yet been realized for various reasons. These include poor engraftment and low transgene expression in the progeny of engineered HScs and the later development of leukaemia in some patients after infusion of engineered HSCs[124]. By contrast, engineered lymphocytes have not suffered from these technical limitations, and investigators have turned increasingly to lymphocyte engineering. Thus, an issue is whether it is safer to use lymphocytes or HSCs for gene transfer therapy. In elegant studies of SCID-X1-immunodeficient patients using gammaretroviral vectors to restore IL-2 receptor γ-chain gene expression in HSCs, investigators showed strong therapeutic efficacy: nine of ten patients were successfully treated, (although, four of the nine developed T cell leukaemia 31-68 months after gene transfer)[125]. At present, no malignancies have been reported in humans following adoptive transfer of genetically engineered T cells. of note is the long incubation period observed before the development of overt leukaemia. we are aware of more than 200 patients receiving treatment as part of various T cell-based trials using gammaretroviral-engineered T cells for cancer, HIV and congenital adenosine deaminase deficiency, in which the patients routinely have detectable engraftment of the engineered T cells for 5 years or longer[56,126,127]. There have been no incidences of transformation reported, and thus as these patients have been engrafted for periods of time that exceed the 'incubation' time required to observe overt transformation in the case of SCID-X1 correction with HSCs, the combined clinical data with engineered T cells supports the notion that gammaretrovirus-based gene transfer therapy with T cells is safe. However, the safety profile could change as the field increasingly turns to the use of engineered lymphocytes that have a more extensive self-renewal capacity.

To experimentally address this issue, von Laer's group[128] transduced mature T cells or HSCs with gammaretroviral vectors expressing the T cell oncogenes *LMO2*, *TCL1* and *TRKA* and then transferred the cells into recombinase-activating gene 1 (*RAG1*)- deficient mice. The animals were followed for at least 1 year and no animals given T cell infusions developed T cell malignancies. By contrast, mice that received an engineered HSC transplant developed T cell lymphoma or leukaemia at a high frequency, leading to the conclusion that polyclonal mature T cells *in vivo* are less susceptible to transformation by known T cell proto-oncogenes than progenitor cells. Studies in mice indicate that there is an inverse relation between clonal frequency and survival of adoptively transferred T cells[129], suggesting that intraclonal competition is a mechanism to maintain a diverse repertoire of T cells and an optimal environment for the generation of long-lived memory cells. One mechanism that may control transformation of engineered T cells is that clonal competition may prevent the emergence of overt leukaemia in mature T cell populations.

Conclusions and perspectives

cell transfer therapy with engineered cells is perhaps the ultimate example of personalized medicine. To become widely available, genetically engineered cells will have to be shown to be clinically effective, scalable, reproducibly manufactured and appropriately priced and marketed. At present, there are formidable challenges in the logistics and costs of goods that

present obstacles to the implementation of therapy with modified cells. However, expensive therapies become economically justified if sufficiently effective. Given that the costs of non-curative antibody therapies and protein replacement therapies frequently exceed US $100,000 per year[130-133], a one-time treatment with engineered cells that may have long-term or even indefinite persistence could be cost effective. Advances in the understanding of lymphocyte biology and cell culture technology, coupled with improved safety and efficiency of genetic modification strategies, have created enthusiasm for several novel adoptive transfer strategies that are now poised for translation into clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Adoptive transfer	A form of immunotherapy in which effector lymphocytes are transfused. Allogeneic adoptive transfer is usually referred to as donor lymphocyte infusion and autologous lymphocyte adoptive transfer is referred to as adoptive transfer therapy.
Therapeutic window	The range of concentrations of a drug, or numbers of immune cells, that will achieve therapeutic effects in most patients with adverse effects in only a small percentage. In cell-based therapies, the condition of the host and the dose and schedule of administration can alter the therapeutic window.
Adeno-associated virus (AAV)	A replication-defective non-enveloped virus that is a member of the *Parvovirus* family. Vectors derived from AAV are attractive because AAV does not cause disease and they can mediate long-term gene transfer in both dividing and non-dividing cells.
Gammaretrovirus	A genus of the *retroviridae* family. Vectors using gammaretroviruses were the first integrating vectors to be used clinically. Most vectors are derived from endogenous retroviruses isolated from mice such as the murine leukaemia virus.
Transposon	Mobile DNA elements that can relocate within the genome of their hosts. Transposons can be used for various applications, including insertional mutagenesis, gene identification, gene tagging and DNA sequencing.
Chimeric antigen receptor	Also known as chimeric immune receptor. Non-MHC-restricted chimeric antigen receptors combine antigen specificity and T cell-activating properties in a single fusion molecule. Most chimeric antigen receptors use an antibody-derived antigen-binding motif to recognize surface-expressed targets, in contrast to T cell receptors, which usually recognize peptide antigens presented by MHC molecules.
MicroRNAs	Single-stranded RNA molecules approximately 21-23 nucleotides in length that are thought to regulate the expression of other genes.
Epigenetic marks	Chemical modifications of chromatin that retain an intact DNA sequence and can modify gene expression. Examples are methylation of cytosine residues and histone modification by acetylation.
Regulatory T (T_{Reg}) cell	A specialized type of $CD4^+$ T cell that can suppress the responses of other T cells. T_{Reg} cells provide a crucial mechanism for the maintenance of peripheral self tolerance and subset of these cells is characterized by the expression of CD25 and FOXP3.
Protein transduction domains (PTDs)	Peptides derived from several viruses, such as the HIV Tat PTD, that can enhance cellular uptake of proteins or polynucleotides. In general, the PTD must be covalently attached to the protein.
Small interfering RNA (siRNA)	Synthetic RNA molecules of 19-23 nucleotides that are used to 'knock down' (that is, silence the expression of) a specific gene.

	This approach is known as RNA interference (RNAi) and is mediated by the sequence-specific degradation of mRNA.
Insertional mutagenesis	Genotoxicity from DNA-based engineering that can result in cellular transformation through various mechanisms. DNA insertion can result in mutations that lead to the activation of oncogenes or to the inactivation of tumour suppressor genes.
Zinc-finger nucleases (ZFNs)	Chimeric proteins comprised of engineered zinc-finger proteins fused to the catalytic domain of a restriction endonuclease that can bind and cleave DNA specifically at a unique and predetermined site in the human genome.
Target product profile	A prospective and dynamic summary of the ideal characteristics of a drug or biological product to ensure that the desired quality and hence the safety and efficacy of a drug product is achieved The target product profile forms the basis of design for the development of the product.
T helper 17 (T_H17) cells	A subset of $CD4^+$ T helper cells that produce interleukin-17 (IL-17) and that are thought to be important in inflammatory and autoimmune diseases. Their generation involves IL-23 and IL-21, as well as the transcription factors RORγt (retinoic-acid-receptor-related orphan receptor-γt) and STAT3 (signal transducer and activator of transcription 3).
Experimental autoimmune encephalomyelitis	An experimental model of multiple sclerosis that is induced by immunization of susceptible animals with myelin-derived antigens, such as myelin basic protein, proteolipid protein or myelin oligodendrocyte glycoprotein.
Central memory T (T_{CM}) cells	Antigen-experienced T cells that express cell surface receptors required for homing to secondary lymphoid organs. These cells are generally thought to be long-lived and can serve as the precursors to effector T cells for recall responses.
Effector memory T (T_{EM}) cells	Terminally differentiated T cells that lack lymph node-homing receptors but express receptors that enable them to home to inflamed tissues. Effector memory T cells can exert immediate effector functions without the need for further differentiation.
$V\gamma9V\delta2^+$ T cells	$V\gamma9V\delta2^+$ T cell receptors are expressed by γδ T cells. $V\gamma9V\delta2^+$ T cells are unique to humans and primates and are a minor fraction of the leukocyte population in peripheral blood (0.5–5%).
SCID mouse	A naturally occurring mouse mutant with severe combined immune deficiency due to an inability to rearrange antigen receptor chain genes.
Natural killer T (NKT) cells	A subpopulation of T cells that expresses both NK cell- and T cell- markers. In the C57BL/6 mouse strain, NKT cells express the NK1.1 (NKRP1C) molecule and the T cell receptor (TCR). Some NKT cells recognize CD1d-associated lipid antigens and express a restricted repertoire of TCRs. After TCR stimulation of naive mice, NKT cells rapidly produce interleukin-4 and interferon-γ.

Replication-competent lentivirus (RCL)	A lentivirus vector that produces infectious virions, RCLs are designed to support only one infections cycle however mutations can induce replication competency.

Box 1

Targeted integration and genetic editing using zinc-finger nucleases

Targeted genetic modification at a predetermined locus in embryonic stem cells has been possible for two decades using homologous recombination[134], enabling the routine creation of genetically targeted mice. However, this approach has not been useful for human gene therapy because the low efficiency of recombination necessitates prolonged cell selection in culture. Zinc-finger nucleases (ZFNs) are chimeric proteins that contain zinc-finger DNA-binding proteins that enable site-specific DNA binding when fused to the catalytic domain of the type IIS restriction enzyme FokI. In essence, ZFNs are designer restriction endonucleases that can cleave DNA specifically at a predetermined genomic target site[135]. The FokI restriction endonuclease is not sequence specific: the target specificity is conferred by the zinc-finger proteins. Once a site-specific double-stranded break is made by FokI, the cell repairs the break using the non-homologous end-joining repair process, which can result in the knockout of the desired gene or the site-specific incorporation of transgene DNA. The potential applications of ZFN technology have been reviewed elsewhere[136,137].

The development of ZFNs has now achieved sufficient efficiency to enable clinical approaches to site-specific genome modification[138]. We have recently reported that the transient expression of ZFNs encoded by plasmid DNA that bind to the HIV-1 co-receptor CC-chemokine receptor 5 *(CCR5)* gene disrupts the locus in approximately 50% of cells, creating cells that are resistant to HIV infection[13]. In collaboration with Sangamo Biosciences we have initiated the first protocol incorporating ZFN technology, testing the adoptive transfer of CCR5-disrupted $CD4^+$ T cells for patients with HIV infection (clinicaltrials.gov indentifier NCT00842634). In this protocol, two ZFNs that bind specific sequences of *CCR5* on opposite strands of DNA are introduced into $CD4^+$ T cells. The cleavage domain of FokI is fused to the 3′ end of each ZFN, which allows dimerization of the FokI cleavage domains, in turn resulting in the generation of a double-stranded break. Repair of the double-stranded break in most cases results in the subsequent disruption of *CCR5* by creating a null allele.

Box 2

Alternative approaches to modify lymphocytes

Important examples of non-virus-based approaches to engineer lymphocytes that leave the genomic DNA sequence intact are listed here.

RnA transduction

mRNA and small interfering RNA (siRNA) can be efficiently introduced into lymphocytes and dendritic cells with low toxicity[22]. For example, the transfer of siRNA designed to downregulate FAS (also known as CD95) expression can render T cells resistant to the apoptotic effects of tumour cells that express FAS ligand (also known as CD95L)[139]. Rossi and colleagues have developed siRNA technology to allow the expression of multiple siRNAs in lymphocytes, so that various combinations of biological effects can be manipulated to generate HIV-resistant cells[19].

DNA methyltransferase inhibitors

The addition of DNA methyltransferase inhibitors to cell culture medium can stabilize the expression and function of forkhead box P3 (FOXP3) in regulatory T (T_{Reg}) cells[36].

Histone deacetylase (HDAC) inhibitors

There are multiple genes that result in hyperacetylation of histones, and multiple agents that inhibit HDACs[39]. Inhibition of HDAC9 enhances T_{Reg}cell function[38].

Protein transduction and painting

The use of protein transduction domains enables the efficient introduction of biologically active cargo into lymphocytes, including proteins and siRNA[42]. Surface engineering of lymphocytes may be used to redirect and alter the function, using strategies that incorporate biotinylation[140] or protein painting[50,141].

Box 3

Vector-specific toxicities due to genetic modification

Gene modification in lymphocytes is most commonly accomplished using gammaretrovirus vectors or lentivirus vectors. The infusion of gene-modified lymphocytes may be associated with the following adverse effects:

- Insertional mutagenesis due to proviral DNA integration, which could lead to clonal proliferation and leukaemia. All forms of genome modification that result in DNA breaks have some risk of oncogenesis. In addition, vector-specific elements such as retroviral long terminal repeats can have genotoxicity[124]. Insertional mutagenesis that resulted in leukaemia has occurred after gene modification of haematopoietic stem cells[142] but has not been reported following modification of mature lymphocytes. Details of the mechanisms of insertional activation have been reviewed elsewhere[120].
- Generation of replication-competent lentiviruses and retroviruses. The retroviral and lentiviral vectors are designed to support only a single round of infection. Early-generation retroviral vectors were susceptible to contamination with helper viruses that produced infectious retroviruses, and these replication-competent retroviruses resulted in T cell lymphoma in rhesus monkeys[143]. Replication-competent lentiviruses can be generated in vitro during vector production by recombination of vector plasmids or in vivo by mobilization of the vector proviral DNA by infectious lentiviruses such as HIV. Conditional replication of a lentiviral vector that was self limiting has occurred during a T cell gene therapy trial[144]; however, the long-term safety of this approach remains to be shown. Under some circumstances, controlled conditional replication of vectors could be of therapeutic benefit[145].
- Germline transmission of the transgene or germline alteration, which could result in vertical transmission to offspring.
- Generation of infectious virus that could be transmitted to other people. This potentially catastrophic event was the subject of a post-apocalyptic book[146] and a recent movie (*I Am Legend*, 2007) about a virologist who uses a recombinant measles virus to cure cancer.

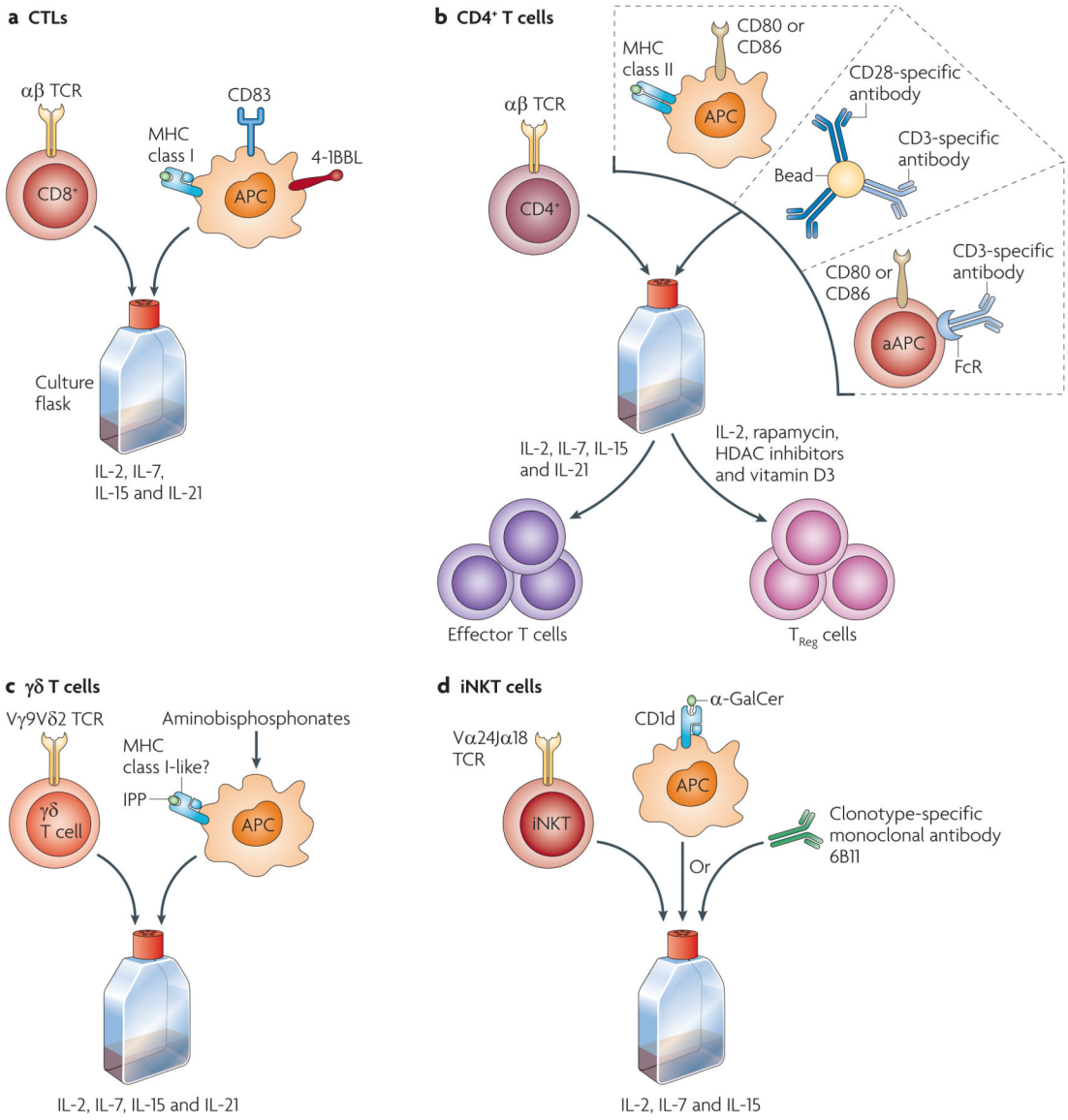

Figure 1. Cell culture approaches for adoptive transfer of lymphocyte subsets

Culture conditions required for distinct lymphocyte subsets vary, depending on the activation and co-stimulatory requirements. **a** | Cytotoxic T lymphocytes (CTLs) express αβ T cell receptors (TCRs) and are stimulated by antigen-presenting cells (APCs) that express MHC class I molecules. CTLs require 4-1BB ligand (4-1BBL; also known as CD137L) stimulation for clonal expansion and have been expanded *in vitro* by the addition of feeder cells or artificial APCs that express the co-stimulatory ligands[147–149]. **b** | $CD4^+$ T cells are stimulated by APCs that express peptide-loaded MHC class II complexes. The main co-stimulatory molecule expressed by $CD4^+$ T cells is CD28, which binds to CD80 or CD86 expressed on APCs. Effector $CD4^+$ T cells can be stimulated by artificial APCs (aAPCs) or beads that bear CD3-specific antibody in the presence of various cytokines[76]. Regulatory $CD4^+$ T (T_{Reg}) cells require culturing in interleukin-2 (IL-2), and the addition of several reagents to the culture may enhance the suppressive functions of *ex vivo* expanded T_{Reg} cells[68]. **c**| Cells expressing γδ TCRs, such as the Vγ9Vδ2 TCR expressed by T cells in the blood, are stimulated by APCs that present exogenous isopentenyl pyrophosphate (IPP) or

other endogenous ligands stimulated by aminobisphosphonates such as zoledronate[86,89]. **d**| CD1d-restricted Vα24Jα 18^+ invaria1nt natural killer T (iNKT) cells are stimulated by α-galactosylceramide (α-GalCer)[99] or the 6B11 clonotype-specific monoclonal antibody[150]. FcR, Fc receptor; HDAC, histone deacetylase.

Table 1
Virus- and non-virus-based approaches for engineering lymphocytes

Approach	Advantages	Disadvantages	use in the clinic	Refs
Virus-based approaches				
Gammaretrovirus vectors	• Extensive safety profile with two decades of clinical use	• Insertional oncogenesis documented after infusion of engineered HSCs • Poor efficiency for modifying other subsets of lymphocytes such as NK cells • Deliver smaller DNA sequences than lentivirus vectors • Susceptible to silencing, particularly in HSCs • Require induction of cell division for integration	Extensive clinical use	2,6,151
Lentivirus vectors	• High transduction efficiencies • Transduce quiescent T cells	• Higher production costs than for gammaretrovirus vectors • Potential genotoxicity owing to LTR sequences and HIV accessory genes; third-generation self-inactivating vectors have improved safety following removal of these sequences	Early stage clinical trials with engineered T cells using VSV-G pseudotyped lentivirus vectors	4,152
Spumavirus vectors or foamy virus vectors	• Non-pathogenic in humans following zoonotic infection • Favourable integration pattern compared with gammaretrovirus and perhaps lentivirus vectors	• Clinical-grade vector manufacturing not yet available	Not yet reached the clinic; foamy virus-mediated insertion of CD18 into HSCs corrected congenital CD18 deficiency in dogs	8,11,153,154
Ad5–F35 vectors	• Recombinant Ad5–35 vectors, which use CD46 as an entry receptor, efficiently infect	• Lymphocytes express low levels of the Ad5 receptor coxsackievirus and adenovirus	Ongoing clinical trial with modified $CD4^+$ T cells for HIV infection (clinicaltrials.gov	12,13,46, 155–157

Approach	Advantages	Disadvantages	use in the clinic	Refs
Virus-based approaches				
	human T cells and support high-level transgene expression for ~1 week • Potential to introduce plasmids and antigens into T cells to generate T cells that can function as APCs	receptor, so are refractory to infection; vector tropism can be modified through manipulation of Ad5 fibre protein	indentifier NCT00842634).	
Lymphotropic herpesvirus (HHV6 and HHV7) vectors	• Infect nearly all humans during childhood, with low pathogenicity • Large payload capacity • Transferred genes expressed efficiently in T cells	• Clinical grade vectors not yet produced	Proposed for use in gene transfer and vaccine therapy	158,159,160
Non-virus-based approaches				
Random integration with electroporation	• Efficient short-term expression of transgenes; rare long-term expression achieved	• For long-term expression, the rare T cells with integrated DNA need to be selected and clonally expanded in culture	Clinical trial with a CD20-specific chimeric immune receptor was successful and resulted in engineered T cells with suboptimal engraftment and antitumour effects	161,162
Transposon-based integration	• A larger payload than retrovirus vectors • High-level, persistent transgene expression • Lower cost than for virus-based vectors	• Tests needed to determine the safety and genotoxicity relative to lentivirus vectors	Not yet entered clinical trials	16,17

Ad, human adenovirus serotype; APC, antigen presenting cell; HHV, human herpes virus; HSC, haematopoietic stem cell; LTR, long terminal repeat; NK, natural killer; VSV-G, vesicular stomatitis virus G.