

REVIEW

Manufacture of tumor- and virus-specific T lymphocytes for adoptive cell therapies

X Wang^{1,2} and I Rivière^{1,2,3}

Adoptive transfer of tumor-infiltrating lymphocytes (TILs) and genetically engineered T lymphocytes expressing chimeric antigen receptors (CARs) or conventional alpha/beta T-cell receptors (TCRs), collectively termed adoptive cell therapy (ACT), is an emerging novel strategy to treat cancer patients. Application of ACT has been constrained by the ability to isolate and expand functional tumor-reactive T cells. The transition of ACT from a promising experimental regimen to an established standard of care treatment relies largely on the establishment of safe, efficient, robust and cost-effective cell manufacturing protocols. The manufacture of cellular products under current good manufacturing practices (cGMPs) has a critical role in the process. Herein, we review current manufacturing methods for the large-scale production of clinical-grade TILs, virus-specific and genetically modified CAR or TCR transduced T cells in the context of phase I/II clinical trials as well as the regulatory pathway to get these complex personalized cellular products to the clinic.

Cancer Gene Therapy (2015) **22**, 85–94; doi:10.1038/cgt.2014.81; published online 27 February 2015

INTRODUCTION

Adoptive cell therapy is an emerging therapeutic platform used to induce tumor regression or clearance of certain viral infections after organ transplantation or hematopoietic stem cell transplantation (HSCT). In addition to virus-specific T cells, two major T-cell sources can confer these therapeutic properties: (1) tumor-infiltrating lymphocytes (TILs) isolated, activated and expanded *ex vivo*; (2) peripheral blood T lymphocytes engineered to express conventional alpha/beta T-cell receptors (TCRs) or tumor-recognizing chimeric antigen receptors (CARs). Clinical cell doses of these autologous tumor-reactive lymphocytes can be manufactured and infused after suitable release testing.^{1–6}

The generation of clinical-grade cellular products encompasses complex processes that are tightly regulated under current good manufacturing practices (cGMP) and requires adequate cell manufacturing facility, ancillary products and manufacturing processes to meet the Food and Drug Administration (FDA) guidelines.⁷ The end-of-process products characteristics such as safety, purity and potency need be carefully defined to meet the quality-control standards.⁸ The manufacturing process needs to be robust and reproducible as well as cost effective. Furthermore, autologous cell therapy products are unique, and the manufacturers must integrate the scientific knowledge defining the product with the FDA regulations. In fact, the regulatory guidelines need be tailored to each individual cell therapy product. In this review, we focus on the large-scale cGMP manufacturing of cells used in adoptive cell therapy including TILs, CAR- and TCR-expressing T cells and viral-specific CTLs.

T-CELL MANUFACTURING APPROACHES

Generation of a therapeutically suitable number of highly active antitumor T cells is a significant technical challenge, and remains

critical for the application of adoptive cell therapy as a standard cancer therapy.

Manufacturing of TILs

Infusion of *ex vivo*-expanded TILs has proven to be a successful treatment regimen for refractory metastatic melanoma.^{9,10} The manufacture of tumor antigen-specific lymphocytes used in adoptive cell transfer is initiated from tumor fragments or single-cell enzymatic digests of resected tumor specimen. A microculture derived from a single tumor fragment or 10^6 viable cells derived from the single-cell enzymatic digestion are placed into one well of a 24-well plate with high dose interleukin-2 (IL-2). Growth medium is changed within 1 week; confluent wells are subsequently split into daughter wells and maintained as independent TIL cultures for generally 1–2 weeks. Cultures are subsequently fed twice per week and maintained at $0.8\text{--}1.6 \times 10^6 \text{ mL}^{-1}$ in flasks. A standard TIL culture typically generates 5×10^7 cells from each original well after 3 to 5 weeks of time. When tumor-reactive TIL cultures are expanded to the minimal requirement of 3×10^7 cells, independent TIL activity and specificity are determined by measuring interferon-gamma secretion by enzyme-linked immunosorbent assay post stimulation with tumor cells. Active individual TIL cultures are then expanded to therapeutic relevant numbers by using a rapid expansion protocol.¹¹ During the rapid expansion phase, 10^6 TIL effector cells are combined with 2×10^8 irradiated, allogeneic healthy donor peripheral blood mononuclear cell (PBMC) feeder cells in presence of anti-CD3 OKT-3 monoclonal antibody (mAb) and high dose IL-2 in tissue culture flasks. Cell density is determined on day 6 of culture and thereafter to maintain a density of 10^5 mL^{-1} by splitting TIL cultures into flasks or culture bags. IL-2 (6000 U mL^{-1}) is used throughout the process to promote cell expansion. Within 2 weeks of time since

¹Cell Therapy and Cell Engineering Facility, Memorial Sloan Kettering Cancer Center, New York, NY, USA; ²Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA and ³Center for Cell Engineering, Memorial Sloan Kettering Cancer Center, New York, NY, USA. Correspondence: Dr I Rivière, Cell Therapy and Cell Engineering Facility, Memorial Sloan Kettering Cancer Center, 1275 York Ave, Box 182, New York, NY 10065, USA.

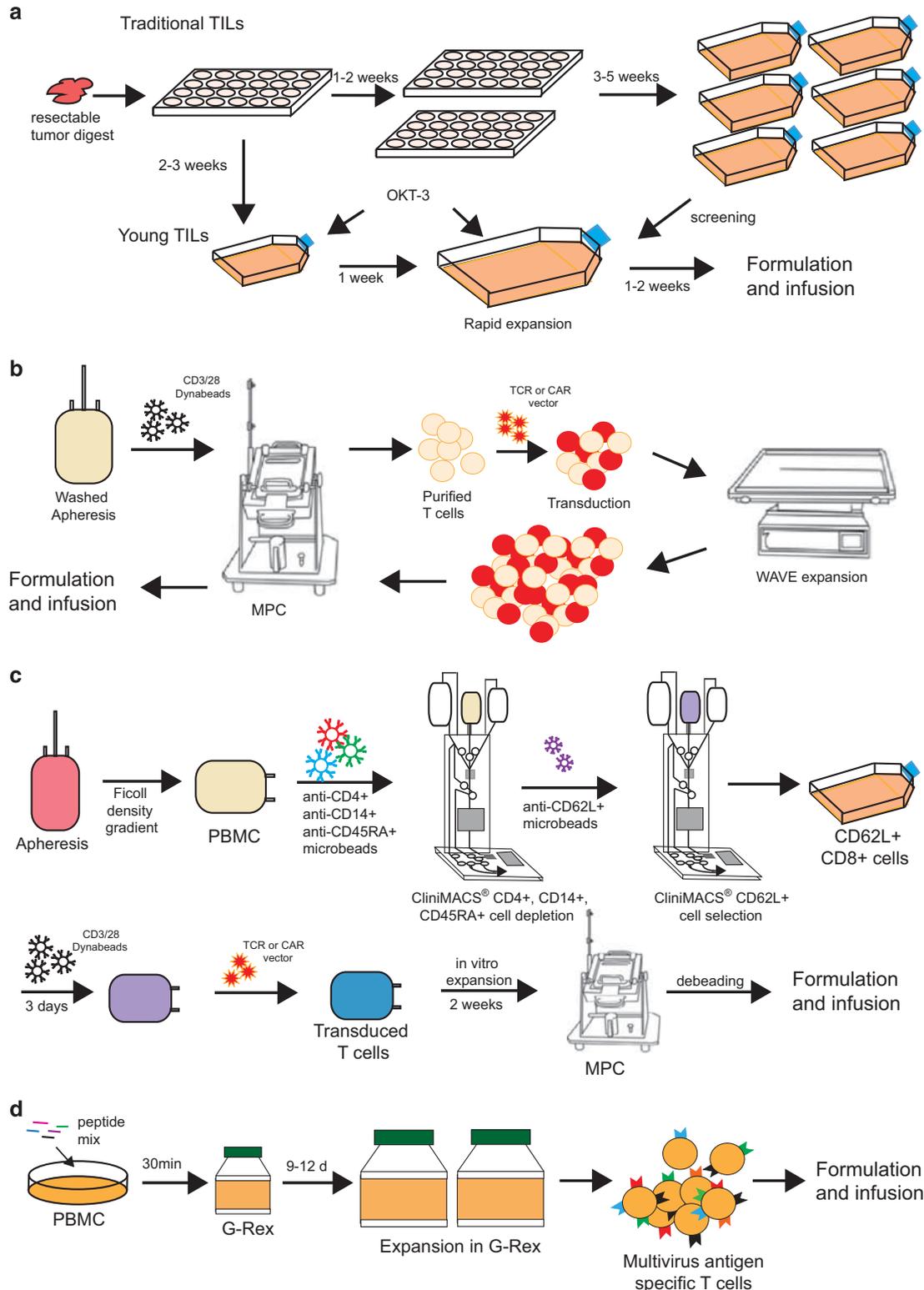
E-mail: rivierei@mskcc.org

Received 21 October 2014; accepted 10 December 2014; published online 27 February 2015

the start of the rapid expansion protocol, cells are harvested, washed, formulated and cryopreserved. The whole manufacture process takes ~6–8 weeks.^{12,13} Products meeting all quality-control tests are released for patient infusion (Figure 1a).

Promising clinical outcomes have been achieved using tumor-reactive TILs in combination with lymphodepletion.¹⁴ However, the extended duration of multiple microcultures and an individualized tumor recognition assay render the process time-

consuming, complex and costly. To circumvent the limitations of such 'standard' method, Dudley and colleagues¹⁵ have simplified and standardized a process to culture 'young' TILs. 'Young' TIL cultures are made of bulk lymphocytes rather than individual microcultures, and the tumor recognition screening assay is eliminated from the process. Young TIL culture is initiated from enzymatic digestion of resected tumor specimen. Single-cell suspension is plated in individual wells of 24-well plates at



5×10^5 cells mL^{-1} in presence of 6000 IU mL^{-1} of IL-2. Five days after initiation, cells are fed and culture media is replaced every 2–3 days thereafter. By day 10 to 18, individual wells of cells are pooled and $\sim 5 \times 10^7$ young TILs are obtained. Rapid expansion of young TILs is performed using the rapid 2-week expansion protocol as described above (Figure 1a). Continuous efforts are made to improve both standard and young TIL manufacturing processes to generate CD8^+ T-cell-enriched culture.¹⁶ More recently, a process to generate epitope-specific TILs by stimulating patient PBMCs with clinical-grade peptides followed by sorting of antigen-specific T cells was published.¹⁷

Manufacturing of T cells genetically engineered to express an exogenous TCR or CAR

Despite the fact that TILs have been shown to mediate antitumor response in 50–70% of melanoma patients, TILs have only limited success in other types of cancers.¹⁸ Furthermore, the generation of TILs is not successful for all melanoma patients.¹⁰ To this end, the genetic modification of peripheral blood lymphocytes to endow these readily accessible cells with antitumor activity is an attractive approach. The power and promise of TCR and CAR-T therapy have been demonstrated by encouraging outcome in patients treated with NY-ESO-1 TCR^{19,20} and CD19-CAR T cells.^{21–24} Many ongoing clinical trials utilized genetically modified T cells, and numerous recent papers have reported their clinical success.²⁵ The key requirement for this genetic modification methodology is the development of RNA vectors expressing TCRs and CARs. TCR can be cloned from the rare occurring patient tumor-reactive T-cell clones,²⁶ from humanized murine models^{27,28} or using the phage display technology.^{29,30} The design of TCR and CAR has steadily improved over the past two decades.^{31–35} For CARs, tumor recognition is mediated by the single-chain variable fragment derived from a monoclonal antibody or humanized Fab. The rationale and strategy of TCR and CAR design and their evolution have been comprehensively reviewed elsewhere.^{36,37}

The manufacture of T cells genetically engineered to express specific TCRs is initiated from Ficoll-purified PBMCs. T cells from PBMCs are activated with OKT-3 antibodies, transduced with a retroviral vector expressing a tumor antigen-specific TCR and cultured for ~ 2 weeks.³⁸ For the CAR-T cells, large-scale transduction and expansion under cGMP has been established,³⁹ and is also applicable to TCR-T cell manufacturing. The process is initiated from the selection and activation of T cells from patient apheresis products using Dynabeads CD3/CD28. $\text{CD3}^+\text{CD28}^+$ T cells are enriched using a magnetic particle concentrator, and are cultured at 10^6 mL^{-1} . The activated T cells are transduced with retroviral vectors in RetroNectin-coated cell bags. The retroviral vector-transduced T cells are inoculated in a WAVE bioreactor on

day 6 to day 8, and expanded with a continuous perfusion regime. By the end of the production run, the beads are removed with the same magnetic bead concentrator and the cells are formulated for infusion either fresh or frozen. The process takes ~ 2 weeks (Figure 1b). This semi-closed large-scale manufacturing platform successfully supports several ongoing clinical trials at MSKCC (NCT01416974, NCT01044069, NCT00466531, NCT01840566, NCT01860937, NCT01140373)^{21,23,39,40} and can be easily adapted for other clinical trials involving the transduction and expansion of autologous or donor T cells.

Other groups are focusing on defining which T-cell subsets are best suited for use in adoptive therapy to generate cell products enriched for these subsets.⁴¹ In animal models, T-cell transfer studies have shown that effector cells from T_{EM} rapidly undergo apoptosis following adoptive transfer and do not persist beyond 7–14 days, whereas a subset of transferred $\text{CD8}^+ \text{T}_{\text{E}/\text{CM}}$ can reacquire memory cell markers, and persist for years.⁴² Consequently, the authors developed a clinical $\text{CD8}^+ \text{T}_{\text{CM}}$ purification, transduction and expansion platform that incorporates clinical scale polyclonal $\text{CD8}^+ \text{T}_{\text{CM}}$ isolation from leukapheresis products, T-cell activation using anti-CD3/CD28 beads without exogenous feeder cells, lentiviral transduction and cell expansion in IL-2/IL-15.⁴² This process is performed with minimal open processing steps and reproducibly yields cryopreserved cell products in excess of 10^9 cells within 35 days (Figure 1c). This platform is currently being used to generate autologous CAR redirected CD19-specific $\text{CD8}^+ \text{T}_{\text{E}/\text{CM}}$ for adoptive transfer following autologous HSCT for high-risk CD19+ non-Hodgkin lymphomas.⁴²

Manufacturing of viral-specific T cells (G-Rex)

Adoptive transfer of viral antigen-specific T cells is a well-established procedure for effective treatment of transplant-associated viral infections and virus-related malignancies. Many laboratories have successfully generated and infused T cells specific for Epstein–Barr virus (EBV), cytomegalovirus and adenovirus using monocytes and EBV-transformed lymphoblastoid cells.^{43–46} Although therapeutic doses of trivirus-specific cytotoxic T lymphocytes can be generated, the original methodology requires 4 to 6 weeks of time for EBV–LCL generation and 4–8 weeks for CTL expansion.^{47,48} Recent process development has been reported for the generation of penta-viral-specific T cells for CMV, AdV, EBV, BK virus (BK) and human herpes virus 6 (HHP6) with dramatically reduced production complexity and time requirement. The process starts with incubation of 1.5×10^7 fresh PBMC and overlapping 15 amino acid peptide mix spanning EBV–LMP2, BZLF1, EBNA1; Adv–Penton, Hexon; CMV–pp65, IE-1; BKV–VP1, large T; HHV6–U11, U14 and U90. The cells are subsequently transferred to G-Rex bioreactors for continuous

Figure 1. Schematic illustration of representative TIL, TCR/CAR-T, and EBV-CTL manufacturing platforms. **(a)** Traditional and young TIL manufacturing scheme. Single-cell digests of resected tumor are plated in 24-well plates as microcultures. For traditional TILs, the microcultures are passaged as independent cultures. Screened and selected TIL cultures are further expanded with a 2-week rapid expansion procedure using OKT-3 antibody. For young TILs, microcultures are pooled without screening. The pooled cells undergo the same 2-week rapid expansion procedure to reach the target dose. During the whole process, cells are maintained in culture with 6000 IU mL^{-1} of IL-2. **(b)** TCR/CAR-T manufacturing process. T cells are selected from washed apheresis product and activated by using CD3/28 Dynabeads and ClinExVivo magnetic particle concentrator (MPC). Activated T cells are transduced with TCR/CAR retroviral or lentiviral vectors, and transduced cells are expanded with WAVE bioreactor. CD3/28 magnetic Dynabeads are removed from the cells with MPC and end of the process cells are formulated for infusion. **(c)** CD8^+ central memory TCR/CAR T-cell manufacturing process. PBMCs are first purified from apheresis product using Ficoll-Plaque gradient centrifugation, followed by CD4^+ , CD14^+ and CD45RA^+ cell depletion using anti-CD4, anti-CD14 and anti-CD45RA microbeads and CliniMACS. Collected cells undergo an additional CD62L positive selection procedure using anti-CD62L microbeads and CliniMACS. Selected $\text{CD8}^+\text{CD62L}^+$ cells are further activated with CD3/28 Dynabeads. Activated memory CD8^+ cells are transduced with TCR/CAR vectors and expanded *in vitro*. Dynabeads are removed from the EOP cells using MCP before formulation. **(d)** Generation of multiviral antigen-specific T cells using G-Rex bioreactor. Donor PBMCs are pulsed with 15mer peptides mix spanning EBV, adenovirus, CMV, BK virus and human herpes virus antigen epitopes and transferred into G-Rex device. Multivirus antigen-specific T cells are expanded to high quantities in ~ 2 weeks of time in the presence of IL-4 and IL-7. CAR, chimeric antigen receptor; CTL, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; IL, interleukin; PBMC, peripheral mononuclear blood cell; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocyte.

culture in presence of IL-4 and IL-7. Therapeutic doses can be achieved in ~10 days of time, in contrast to the 10-week period required with the traditional approach. Monovalent-, bivalent-, trivalent-, tetravalent- and pentavalent-specific T-cell products are efficiently generated with this method, whereas the range of antiviral activity is limited by the previous viral exposure of the donor T cells. The multiviral-specific T-cell lines generated using this method have been demonstrated to have up to a 94% response rate in post HSCT patient with viral infections⁴⁹ (Figure 1d). Viral-specific T cells can be further genetically engineered to express TCRs and CARs to have a second specificity for tumor antigens.^{44,50–52} A GMP manufacture process has also been recently tested, in which the CliniMACS-purified viral-specific T cells were transduced with retroviral vectors, and expanded *in vitro*.⁵³

To broaden the use of CAR-modified T cells that could provide a GVL effect after allo-HSCT without concomitant GVHD, Riddell *et al.*⁴¹ and others⁵⁴ have proposed to combine the use of viral-specific T cells such as CMV- and EBV-specific CD8⁺ T cells to generate CAR expressing T cells derived from central memory T (T_{CM}) cells as they are deemed capable of persisting long term.⁵⁵ In one method, the CD45RA⁺CD8⁺ cell fraction is enriched by depletion of CD4⁺, CD14⁺ and CD45RA⁺ cells on the CliniMACS device using clinical-grade mAbs and paramagnetic beads.⁴² The CD62L⁺ cells are subsequently enriched by positive selection with a clinical-grade biotin-conjugated anti-CD62L mAb and anti-biotin microbeads. In brief, the enriched CD8⁺CD62L⁺ T cells are plated with either autologous γ -irradiated peptide-pulsed PBMCs or monocyte-derived dendritic cells in 50 IU mL⁻¹ IL-2. On day 1 after stimulation, the T cells are exposed to lentiviral vector stocks encoding the CD19-CAR in presence of polybrene followed by spinoculation. After 8–10 days in culture, the cells are pooled and analyzed by flow cytometry after staining with virus-specific human leukocyte antigen tetramers. The transduced T cells are expanded in culture by plating with γ -irradiated LCLs and fed with 50 IU mL⁻¹ IL-2. After 10–14 days of culture, cells are stained with virus-specific human leukocyte antigen tetramers and Abs specific for transduction markers. The virus-specific subset of transduced T cells is then purified using reversible class I MHC streptamers. The selected cells can also be transduced with a lentiviral vector CAR transgene modified to co-express a truncated version of the epidermal growth factor receptor that can be detected by biotinylated anti-EGFR (Erbixut) mAb.^{56–58}

EXPRESSION VECTORS FOR GENETIC MODIFICATION OF T CELLS

Three main types of gene expression vectors are currently used in clinical applications for TCR and CAR delivery in T cells. They include gamma retroviral vectors, lentiviral vectors and transposons. We will focus herein on the large-scale manufacturing platforms for these critical reagents.

Gamma-retroviral vector

Gamma-retroviral vectors were the first viral vectors used for clinical application.⁵⁹ They are still used as gene-transfer vehicles in about 20% of the current clinical trials.⁶⁰ The wide usage of gamma retroviral vectors is due to their broad cell tropism, efficient integration and stable gene expression in target cells. In addition, they can be consistently manufactured at relatively low cost. Many stable packaging cell lines, such as PA317,⁶¹ PG13⁶² and 293GP,⁶³ have been developed. We share with several groups the combinatorial use of the SFG vector and PG13 packaging cell line.^{23,44,64–66} The clonal selection and expansion of high-titer-producer cells can yield the desired stable gamma retroviral cell clone. Subsequently, a master cell bank of the stable packaging cell clone can be generated and qualified. Large-scale

manufacturing protocols have been reported from different laboratories. The manufacturing process starts from the expansion of stable producer cells in roller bottles,^{67,68} 10-layer cell factories⁶⁴ or bioreactors⁶⁹ (Figure 2a). Gamma-retroviral vectors cannot be harvested longer than three consecutive days due to the relative short half-life of gamma retroviral vectors. The harvests are pooled at the end of the production run and filtered using a step-filtration step to efficiently remove packaging cell contaminants from the vector stocks.^{64,67} The vector stocks are aliquoted and frozen in vials or cryobags. In the case of gamma retroviral vectors with a self-inactivating design, vector manufacturing relies on transient transfection-based techniques similar to lentiviral vector production.⁷⁰

To release the vector stocks for clinical use, a series of biosafety testing is required, which include but are not limited to sterility on end-of-process cells (EOP) and final product vector stocks (FP), mycoplasma testing (EOP and FP), general safety (FP), transmission electron microscopy (EM) on bulk vector stocks, *in vitro* adventitious virus testing (FP) and GalV replication-competent retrovirus (GalV RCR; EOP and FP).³⁹ The production of gamma retroviral vectors in serum-free media or media containing serum replacement is highly desirable for clinical trials beyond phase I but remains a challenge.^{71,72} Gamma-retroviral vectors have been shown to be safe in patients who received T cells genetically modified to express LNGF-R, HSV-TK, neomycin, adenosine deaminase or an anti-HIV-1 *tat* ribozyme. After up to 10 years follow-up, these patients have not developed any evidence of T-cell clonal expansion.^{73–76}

Lentiviral vector

Lentiviral vectors have been successfully utilized to engineer hematopoietic stem cells for the treatment of adrenoleukodystrophy,⁷⁷ beta-thalassemia,^{78,79} Wiskott–Aldrich syndrome⁸⁰ and metachromatic leukodystrophy⁸¹ as well as CAR T cells for hematologic diseases.^{22,56,82} Similar to gamma retroviral vectors, lentiviral vectors mediate efficient gene transfer and high level of transgene expression. The commonly used VSV-G pseudo envelope also endows broad tropism. Compared with gamma retroviral vectors, lentiviral vectors display several favorable features such as the ability to transduce nondividing cells^{83–85} and relative safer chromosome integration profile,⁸⁶ it should be noted that gamma retroviral vectors have not been reported to be genotoxic in terminally differentiated cells such as T lymphocytes.^{73–76} Significant hurdles in production and purification processes to obtain sufficient quantities of GMP grade lentiviral vector stocks for phase I clinical trials and beyond need to be overcome. Stable producer cell lines are difficult to generate and are not widely available for lentiviral vector production.^{87,88} The commonly used manufacturing platforms for the third- and fourth-generation packaging systems are based on transient transfection of three or four independent plasmids encoding gag-pol-rev, the self-inactivating transfer vector and the pseudo envelope. For the fourth-generation packaging system, the rev gene can also be encoded on a separate plasmid. HEK293 cell and its derivatives such as 293T,⁸⁹ 293E⁹⁰ are the principle cell lines used for lentiviral vector production. The calcium phosphate precipitation method is traditionally used for transfection. Another cost-effective compound, polyethylenimine, has also been qualified and used in recent years^{91,92} as well as flow electroporation.⁹³ Other lipid-based methods are still too expensive to be used in a large-scale manufacturing setting. For large-scale lentiviral vector production, HEK293-derived cells are expanded in large quantity. The method of culture expansion is a critical component for generating vector stocks with high titer and yield. The available scalable expansion systems include the cell factory system, the HYPERFlask, microcarriers and bioreactors.^{70,94,95} The downstream processes for

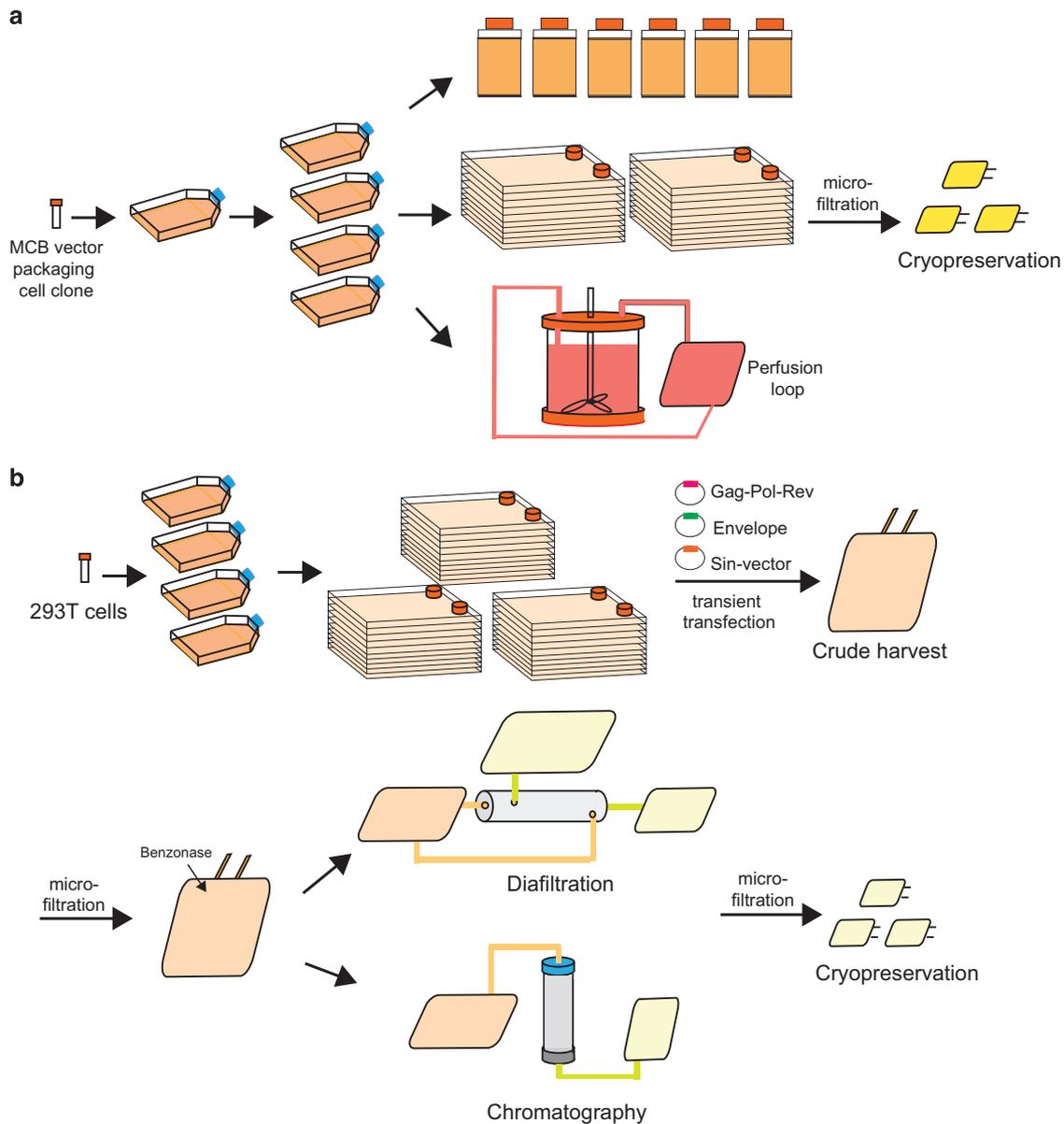


Figure 2. Retroviral and lentiviral vectors' manufacturing platform. **(a)** Generation of retroviral vector. High titer-producer cells from master cell bank are thawed and expanded in T flasks. Cells are further expanded in either roller bottles, cell factories or bioreactors. Vector stocks are harvested in the optimized harvesting window, filtered to removed contaminants and cryopreserved for biosafety testing before release for clinical use. **(b)** Manufacturing of lentiviral vector using transient transfection in 10-layer cell factories. 293T cells or derivatives are expanded to large quantity to inoculate multiple 10-layer cell factories. Cells are transiently transfected with packaging, envelope, and SIN-vector plasmids. Crude vector stocks are harvested and filtered. Benzonase is added to the crude harvests to remove plasmid contaminants. Vector stocks need to be further purified and concentrated using diafiltration or chromatography methodologies. Purified and concentrated vector stocks are cryopreserved for biosafety testing to be qualified for clinical use. MCB, master cell bank; SIN, self-inactivating.

lentiviral vector production aims at removing cell and plasmid contaminants, concentrating vector particles to achieve high titer vector stocks while maintaining vector potency. These are challenging tasks that typically encompass the following steps: (1) Vector stocks harvesting. Owing to the nature of transient transfection, crude lentiviral vector stocks can be harvested for 2 days. Generally, the titer of the vector stocks beyond 2-day harvest is too low to be used; (2) Clarification. This step is to eliminate producer cells and cell debris from the crude harvest. It can be achieved by centrifugation or dead-end filtration. Microfiltration is needed to achieve greater clarification for downstream ultrafiltration or chromatography; (3) Nucleic acid

digestion. Plasmid DNAs used for transfection are the major source of DNA contaminants. Cellular DNA and RNA may also be released during cell culture. Nucleic acids need to be removed to meet safety requirements and decrease sample viscosity, a major cause of column clotting. Benzonase is commonly used for this purpose; (4) Concentration and purification. Ultracentrifugation is the most widely used method for lentiviral vector concentration in a research setting. Ultrafiltration and chromatography are the preferred methods for manufacturing under cGMP. Although different filtration modes and devices are available for ultrafiltration, tangential-flow filtration is the most widely used method for its effectiveness and better yield. Chromatography is another

Table 1. Quality-control assays for clinical-grade retroviral and lentiviral vectors

	Testing	Example assays	Criteria
Purity	Lentiviral vector		
	Total proteins (ng mL ⁻¹)	ELISA	Report results
	Bovine serum albumin (ng mL ⁻¹)	ELISA	Report results
	Benzonase (ng mL ⁻¹)	ELISA	Report results or < 100 ng mL ⁻¹
	Plasmid DNA (copies per 100 ng)	VSVg qPCR optional or serial washes	Below detection or decrease over time
Safety	Host cell specific DNA (copies per 100 ng)	qPCR	Report results
	SV40 LTA and E1A qPCR	qPCR	Below detection
	Retroviral and lentiviral vector		
	Sterility	USP, No growth for 14 days	No growth for 14 days
	Mycoplasma	culture on Vero indicator, PTC	Negative
Potency	Endotoxin/pyrogen	LAL	< 10 EU mL ⁻¹
	<i>In vitro</i> Adventitious agents	Assay on MRC5, Vero and A549 cells	Negative
	Retroviral vector		
	RCR	Marker-rescue cell culture assay	No RCR detected
	General safety (first lot)	Current USP	Absence of adverse agents
Others	Lentiviral vector		
	RCL	Co-culture on C8166 cells with amplification and indicator phases	No RCL detected
	Retroviral and lentiviral vector		
	Infectious viral particles	Gene transfer/expression assay in cell line of choice	Report results
	Retroviral vector		
Others	Total viral particles	EM	Only type C retroviral like particles detected
	Lentiviral vector		
	Total viral particles	p24 ELISA, qRT-PCR	Report value
	Retroviral and lentiviral vector		
	Physicochemical characteristics	pH (optional) Appearance	6.9–7.8 Opaque

Abbreviations: ELISA, enzyme-linked immunosorbent assay; LAL, limulus amoebocyte lysate; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; RCL, replication competent lentivirus; RCR, replication competent retrovirus; USP, U.S. pharmacopeial convention.

preferred method for GMP manufacturing. A number of chromatography methods, including anion exchange chromatography, affinity chromatography and size exclusion chromatography⁹⁶ have been reported for the purification of lentiviral vector particles; (5) Sterile filtration and storage. Membrane filtration through 0.22 µm pores is the last step in the generation of clinical-grade lentiviral vector (Figure 2b). Vectors are packaged and stored in -80 °C and a series of quality-control assays are performed before release for clinical use (Table 1). Similar to gamma retroviral vectors, the production in serum-free media is desirable for clinical trials beyond phase I but remains challenging.⁹⁷ The production of lentiviral vectors has been comprehensively reviewed by Schweizer and Merten.⁹⁸

Sleeping beauty transposon/transposase system

Transposon/transposase is a relatively new expression system in the gene therapy field. It is a nonviral, plasmid-based methodology. The transposon/transposase system is derived from fish and has been adapted for gene therapy. The sleeping beauty (SB) system consists of two DNA plasmids: one plasmid is the transposon that encodes the gene of interest, such as CAR or TCR; the second plasmid expresses the transposase that enables the insertion of the transgene into TA dinucleotide repeats. The SB transposon/transposase have been used to produce genetically modified CAR-T cells for phase I/II clinical trial,^{99,100} in which SB transposon/transposase are introduced into T cells by electroporation. Transfected T cells are subsequently expanded on artificial antigen-presenting cells.¹⁰¹ The advantages of using the SB system are that the clinical-grade plasmids are much simpler to produce, and the cost effectiveness due to lesser safety testing requirements

when compared to cell products genetically modified with gamma retroviral or lentiviral vectors (Table 2).

PROCESS VALIDATION

The bench-to bedside transition for innovative adaptive cell therapy requires carefully designed scale-up and validation processes. Process validation is required to establish scientific evidence that a process is capable of consistently delivering quality products. FDA issued new guidelines for process validation in 2011.¹⁰² Process validation can be broken down into the following three stages.

Process design stage

Process design is based on the knowledge gained through process development and scale-up activities, including those gained from research laboratories, process engineering, pilot and small-scale studies. The goal of this stage is to design a process suitable for routine manufacturing procedures. Early process design experiments do not need to be performed under cGMP; however, maintaining detailed records of reagents and procedures is highly advisable. During this stage, maintaining the right balance between process complexity and practicality is important to ensure broad downstream application. The selection of reagents that allows freedom to operate can be a challenge as these therapies demonstrate promising outcomes.

Table 2. Example of Quality-control assays for clinical-grade SB plasmid

	Testing	Example assays	Criteria
Purity	Identity	Restriction mapping and agarose gel	Expected band size
	Sequencing	Sanger, high throughput	Confirm the original coding sequence
	Concentration	absorbance at 260nm	$2.0 \pm 0.2 \text{ mg mL}^{-1}$
Safety	A260/A280 absorbance	assay name	1.8 ± 0.2
	Plasmid Form	HPLC	> 90% supercoiled
	Sterility Test	USP, no growth within 14 days	No growth for 14 days
	Bacterial endotoxin	Kinetic LAL test	< 50 EU mg^{-1}
	<i>E. coli</i> host protein	ELISA	< 0.3%
Others	<i>E. coli</i> RNA	HPLC	< 10%
	Appearance	Observation	Clear, colorless, liquid

Abbreviations: *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; SB, Sleeping Beauty.

Process qualification stage

During this stage, the process design is evaluated to determine whether it is performing in the intended manner. This stage includes two elements: (1) Facility design as well as equipment and utility qualification; and (2) process performance qualification. Current GMP-compliant procedures must be followed at this stage.¹⁰³ Equipment and utility qualification can either be performed under individual plans or as part of an overall project plan. The quality-control unit must review and approve the qualification plan and report. The process performance qualification combines the actual facility, utilities, equipment and trained personnel with the manufacturing process. A written protocol specifying the manufacturing conditions, controls, testing and expected outcome is essential at this stage. In most cases, process performance qualifications have a higher level of sampling, additional testing and greater scrutiny. A performance analysis report should be prepared in a timely fashion post completion of the process. The successful execution of process performance qualification is a major step in the product life cycle.

Continued process verification

This stage of validation ensures that the manufacturing process remains in a state of control. The equipment and facility qualification status must be maintained through routine monitoring, maintenance and calibration procedures.

Data generated during processes related to product quality need to be collected and analyzed in a timely fashion by qualified individuals. These results help the manufacturers gain deeper understanding of the source of variability, the presence and degree of variation and the impact of these variations on the process and product. A change /optimization of the process may be warranted based on the data collected.

Documentation at all stages of process validation life cycle is essential for effective communication in the complicated, lengthy, and multidisciplinary process of cell manufacturing. Quality is built in the product, not solely tested in the final product. An open and ongoing dialogue between manufacturing team and quality assurance/quality control teams is the key for establishing a successful manufacturing platform.

RELEASE TESTING AND CERTIFICATE OF ANALYSIS

An appropriate set of practical and scientifically defensible release criteria is essential to guarantee the products' integrity, consistency and efficacy. The underlying principle for release criteria is to provide adequate testing to ensure the product identity, purity, safety and potency. The cellular identity of T-cell products is commonly characterized by cell surface marker expression detected by flow cytometry analysis. It can also include

more defined cell subset composition⁴² or residual tumor contamination. The interpretation of purity here means lack of endotoxin or other potential harmful materials contaminating the product during manufacturing. Safety of the product requires that it is sterile and free of mycoplasma contamination and of RCR or RCL. Cellular products need to be viable (generally $\geq 70\%$), and genetically modified cellular products may need to reach a minimum transduction efficiency as a potency criteria.

Sterility is a fundamental test required for the release of cellular products. Standard sterility tests described in 21CFR610.2 for bacterial and fungal contamination requires 14 days of incubation. Bactec automated-based method are also being considered and can be validated for cultured cell products.^{104,105} When only short time intervals are foreseeable between completion of manufacturing and product release, Gram staining can be used in combination with sterility results on in process samples collected 24 and 48 h before formulation. 'Points to Consider' is the method recommended by the FDA for mycoplasma testing for all *ex vivo* cultures. Although there are other commercially available PCR-based kits to detect this contaminant, these methods are not approved by the regulatory agency. They may be used if they are properly validated during the process validation. A rapid release assay for endotoxin has been developed using the Endosafe PTS endotoxin device, which takes about 20 min and is approved by the FDA.¹⁰⁶ Viability assessment of cells is a routine requirement that can be done by various methods, including trypan blue exclusion, 7-aminoactinomycin D staining coupled with flow cytometric analysis, and acridine orange and propidium iodide staining followed by automatic cell counting. Other required product-specific assays should be established earlier on in the process development phase and approved by FDA under the investigational new drug application.

The release of the cell product for infusion is handled through the issuance of a certificate of analysis (C of A). The C of A summarizes the characteristics of the product and the tests performed. The C of A also details the release specifications and results of each test including the method used, assay sensitivity and acceptable range of results. Example of released tests used for CAR-T cells were previously published by our group³⁹ and by others.^{82,101}

CONCLUSION

Treating cancers by harnessing the power of the immune system holds great promise for future cancer therapies. Cumulative evidence shows that adoptive T-cell therapy is an effective treatment for various tumors, including melanoma, hematological cancers and some viral infections post organ transplantation and HSCT. Yet, breakthroughs are still awaited in the field of solid

tumors. TILs and the genetically modified TCR and CAR transduced T cells as therapeutic modalities are progressing toward a more mature stage. Although autologous cell therapy poses unprecedented challenges in terms of manufacturing and distribution for commercialization purposes, TCR- and CAR-transduced T cells recently became part of the portfolio of biotechnology and large pharmaceutical companies. Multiple partnerships between academic centers and industry have been established.¹⁰⁷ As a result, improved and semi-automated manufacturing platforms are likely to be developed that will allow wide dissemination of these promising therapies and will encourage novel research approaches. Adoptive T-cell therapies are poised to become part of the standard of care treatments for patients with cancer.

CONFLICT OF INTEREST

IR is a scientific cofounder of and a consultant for Juno Therapeutics. XW declares no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Michel Sadelain for critically reviewing the manuscript. This work is supported by NCI P30 CA08748, P50 CA086438.

REFERENCES

- Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 2008; **8**: 299–308.
- Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol* 2012; **12**: 269–281.
- Humphries C. Adoptive cell therapy: honing that killer instinct. *Nature* 2013; **504**: S13–S15.
- Maus MV, Fraietta JA, Levine BL, Kalos M, Zhao Y, June CH. Adoptive immunotherapy for cancer or viruses. *Annu Rev Immunol* 2014; **32**: 189–225.
- Yee C. The use of endogenous T cells for adoptive transfer. *Immunol Rev* 2014; **257**: 250–263.
- Davila ML, Brentjens R, Wang X, Riviere I, Sadelain M. How do CARs work?: Early insights from recent clinical studies targeting CD19. *Oncoimmunology* 2012; **1**: 1577–1583.
- FDA. 'Guidance for industry: current good manufacturing practice for blood and blood components: (1). Quarantine and disposition of units from prior collections from donors with repeatedly reactive screening test for antibody to hepatitis C virus (anti-HCV); (2) Supplemental testing, and the notification of consignees and blood recipients of donor test results for anti-HCV; availability—FDA. Notice. *Fed Regist* 1998; **63**: 56198–56199.
- Gee AP. Product release assays. *Cytotherapy* 1999; **1**: 485–491.
- Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ *et al*. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002; **298**: 850–854.
- Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U *et al*. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008; **26**: 5233–5239.
- Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J Immunol Methods* 1990; **128**: 189–201.
- Topalian SL, Muul LM, Solomon D, Rosenberg SA. Expansion of human tumor infiltrating lymphocytes for use in immunotherapy trials. *J Immunol Methods* 1987; **102**: 127–141.
- Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. *J Immunother* 2003; **26**: 332–342.
- Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy. *Sci Transl Med* 2012; **4**: 127ps8.
- Tran KQ, Zhou J, Dürflinger KH, Langhan MM, Shelton TE, Wunderlich JR *et al*. Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. *J Immunother* 2008; **31**: 742–751.
- Dudley ME, Gross CA, Langhan MM, Garcia MR, Sherry RM, Yang JC *et al*. CD8+ enriched 'young' tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* 2010; **16**: 6122–6131.
- Labarriere N, Fortun A, Bellec A, Khammari A, Dreno B, Saiagh S *et al*. A full GMP process to select and amplify epitope-specific T lymphocytes for adoptive immunotherapy of metastatic melanoma. *Clin Dev Immunol* 2013; **2013**: 932318.
- Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* 2014; **257**: 56–71.
- Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, Dudley ME *et al*. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011; **29**: 917–924.
- Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R *et al*. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 2008; **358**: 2698–2703.
- Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG *et al*. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* 2013; **5**: 177ra38.
- Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR *et al*. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* 2013; **368**: 1509–1518.
- Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K *et al*. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* 2014; **6**: 224ra25.
- Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M *et al*. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *J Clin Oncol* 2014. (e-pub ahead of print).
- Aranda F, Vacchelli E, Obrist F, Eggermont A, Galon J, Herve Fridman W *et al*. Trial watch: adoptive cell transfer for anticancer immunotherapy. *Oncoimmunology* 2014; **3**: e28344.
- Johnson LA, Heemskerck B, Powell DJ Jr., Cohen CJ, Morgan RA, Dudley ME *et al*. Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *J Immunol* 2006; **177**: 6548–6559.
- Parkhurst MR, Joo J, Riley JP, Yu Z, Li Y, Robbins PF *et al*. Characterization of genetically modified T-cell receptors that recognize the CEA:691-699 peptide in the context of HLA-A2.1 on human colorectal cancer cells. *Clin Cancer Res* 2009; **15**: 169–180.
- Cohen CJ, Zheng Z, Bray R, Zhao Y, Sherman LA, Rosenberg SA *et al*. Recognition of fresh human tumor by human peripheral blood lymphocytes transduced with a bicistronic retroviral vector encoding a murine anti-p53 TCR. *J Immunol* 2005; **175**: 5799–5808.
- Li Y, Moyssey R, Molloy PE, Vuidepot AL, Mahon T, Baston E *et al*. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat Biotechnol* 2005; **23**: 349–354.
- Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG *et al*. Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nat Med* 2008; **14**: 1390–1395.
- Baum C, Schambach A, Bohne J, Galla M. Retrovirus vectors: toward the plentivirus? *Mol Ther* 2006; **13**: 1050–1063.
- Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z *et al*. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *J Immunol* 2008; **180**: 6116–6131.
- Cohen CJ, Zhao Y, Zheng Z, Rosenberg SA, Morgan RA. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 2006; **66**: 8878–8886.
- Cohen CJ, Li YF, El-Gamil M, Robbins PF, Rosenberg SA, Morgan RA. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. *Cancer Res* 2007; **67**: 3898–3903.
- Voss RH, Willemsen RA, Kuball J, Grabowski M, Engel R, Intan RS *et al*. Molecular design of the Calphabeta interface favors specific pairing of introduced TCRalphabeta in human T cells. *J Immunol* 2008; **180**: 391–401.
- Suerth JD, Schambach A, Baum C. Genetic modification of lymphocytes by retrovirus-based vectors. *Curr Opin Immunol* 2012; **24**: 598–608.
- Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. *Cancer Discov* 2013; **3**: 388–398.
- Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM *et al*. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**: 126–129.
- Hollyman D, Stefanski J, Przybylowski M, Bartido S, Borquez-Ojeda O, Taylor C *et al*. Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. *J Immunother* 2009; **32**: 169–180.

- 40 Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J *et al.* Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 2011; **118**: 4817–4828.
- 41 Riddell SR, Sommermeyer D, Berger C, Liu LS, Balakrishnan A, Salter A *et al.* Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. *Cancer J* 2014; **20**: 141–144.
- 42 Wang X, Naranjo A, Brown CE, Bautista C, Wong CW, Chang WC *et al.* Phenotypic and functional attributes of lentivirus-modified CD19-specific human CD8+ central memory T cells manufactured at clinical scale. *J Immunother* 2012; **35**: 689–701.
- 43 Koehne G, Smith KM, Ferguson TL, Williams RY, Heller G, Pamer EG *et al.* Quantitation, selection, and functional characterization of Epstein-Barr virus-specific and alloreactive T cells detected by intracellular interferon-gamma production and growth of cytotoxic precursors. *Blood* 2002; **99**: 1730–1740.
- 44 Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G *et al.* Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008; **14**: 1264–1270.
- 45 Doubrovina E, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J *et al.* Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood* 2012; **119**: 2644–2656.
- 46 Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA *et al.* Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 2010; **115**: 925–935.
- 47 Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA *et al.* Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* 1995; **345**: 9–13.
- 48 Smith CA, Ng CY, Heslop HE, Holladay MS, Richardson S, Turner EV *et al.* Production of genetically modified Epstein-Barr virus-specific cytotoxic T cells for adoptive transfer to patients at high risk of EBV-associated lymphoproliferative disease. *J Hematother* 1995; **4**: 73–79.
- 49 Papadopoulos A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C *et al.* Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. *Sci Transl Med* 2014; **6**: 242ra83.
- 50 Rossig C, Bollard CM, Nuchtern JG, Rooney CM, Brenner MK. Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. *Blood* 2002; **99**: 2009–2016.
- 51 Heemskerk MH, Hoogeboom M, Hagedoorn R, Kester MG, Willemze R, Falkenburg JH. Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. *J Exp Med* 2004; **199**: 885–894.
- 52 Savoldo B, Rooney CM, Di Stasi A, Abken H, Hombach A, Foster AE *et al.* Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30zeta artificial chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood* 2007; **110**: 2620–2630.
- 53 van Loenen MM, de Boer R, van Liemp E, Meij P, Jedema I, Falkenburg JH *et al.* A Good Manufacturing Practice procedure to engineer donor virus-specific T cells into potent anti-leukemic effector cells. *Haematologica* 2014; **99**: 759–768.
- 54 Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD *et al.* Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* 2011; **118**: 6050–6056.
- 55 Wang X, Berger C, Wong CW, Forman SJ, Riddell SR, Jensen MC. Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. *Blood* 2011; **117**: 1888–1898.
- 56 Wang X, Chang WC, Wong CW, Colcher D, Sherman M, Ostberg JR *et al.* A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and ablation of engineered cells. *Blood* 2011; **118**: 1255–1263.
- 57 Neudorfer J, Schmidt B, Huster KM, Anderl F, Schiemann M, Holzappel G *et al.* Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* 2007; **320**: 119–131.
- 58 Terakura S, Yamamoto TN, Gardner RA, Turtle CJ, Jensen MC, Riddell SR. Generation of CD19-chimeric antigen receptor modified CD8+ T cells derived from virus-specific central memory T cells. *Blood*. 2012; **119**: 72–82.
- 59 Rosenberg SA, Aebbersold P, Cornetta K, Kasid A, Morgan RA, Moen R *et al.* Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 1990; **323**: 570–578.
- 60 Deichmann A, Schmidt M. Biosafety considerations using gamma-retroviral vectors in gene therapy. *Curr Gene Therapy* 2013; **13**: 469–477.
- 61 Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 1986; **6**: 2895–2902.
- 62 Miller AD, Garcia JV, von Suhr N, Lynch CM, Wilson C, Eiden MV. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J Virol* 1991; **65**: 2220–2224.
- 63 Ghani K, Cottin S, Kamen A, Caruso M. Generation of a high-titer packaging cell line for the production of retroviral vectors in suspension and serum-free media. *Gene Ther* 2007; **14**: 1705–1711.
- 64 Przybylowski M, Hakakha A, Stefanski J, Hodges J, Sadelain M, Riviere I. Production scale-up and validation of packaging cell clearance of clinical-grade retroviral vector stocks produced in cell factories. *Gene Ther* 2006; **13**: 95–100.
- 65 Gallardo HF, Tan C, Ory D, Sadelain M. Recombinant retroviruses pseudotyped with the vesicular stomatitis virus G glycoprotein mediate both stable gene transfer and pseudotransduction in human peripheral blood lymphocytes. *Blood* 1997; **90**: 952–957.
- 66 Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G *et al.* CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest* 2011; **121**: 1822–1826.
- 67 Reeves L, Cornetta K. Clinical retroviral vector production: step filtration using clinically approved filters improves titer. *Gene Ther* 2000; **7**: 1993–1998.
- 68 Feldman SA, Goff SL, Xu H, Black MA, Kochenderfer JN, Johnson LA *et al.* Rapid production of clinical-grade gammaretroviral vectors in expanded surface roller bottles using a 'modified' step-filtration process for clearance of packaging cells. *Hum Gene Ther* 2011; **22**: 107–115.
- 69 Merten OW, Cruz PE, Geny-Fiamma C, Bouquet C, Goncalves D *et al.* Comparison of different bioreactor systems for the production of high titer retroviral vectors. *Biotechnol Prog* 2001; **17**: 326–335.
- 70 van der Loo JC, Swaney WP, Grassman E, Terwilliger A, Higashimoto T, Schambach A *et al.* Scale-up and manufacturing of clinical-grade self-inactivating gamma-retroviral vectors by transient transfection. *Gene Ther* 2012; **19**: 246–254.
- 71 Rodrigues AF, Carmo M, Alves PM, Coroadinha AS. Retroviral vector production under serum deprivation: The role of lipids. *Biotechnol Bioeng* 2009; **104**: 1171–1181.
- 72 Ghani K, Wang X, de Campos-Lima PO, Olszewska M, Kamen A, Riviere I *et al.* Efficient human hematopoietic cell transduction using RD114- and GALV-pseudotyped retroviral vectors produced in suspension and serum-free media. *Hum Gene Ther* 2009; **20**: 966–974.
- 73 Bonini C, Grez M, Traversari C, Ciceri F, Marktel S, Ferrari G *et al.* Safety of retroviral gene marking with a truncated NGF receptor. *Nat Med* 2003; **9**: 367–369.
- 74 Brenner MK, Heslop HE. Is retroviral gene marking too dangerous to use? *Cytotherapy* 2003; **5**: 190–193.
- 75 Macpherson JL, Boyd MP, Arndt AJ, Todd AV, Fanning GC, Ely JA *et al.* Long-term survival and concomitant gene expression of ribozyme-transduced CD4+ T-lymphocytes in HIV-infected patients. *J Gene Med* 2005; **7**: 552–564.
- 76 Muul LM, Tuschong LM, Soenen SL, Jagadeesh GJ, Ramsey WJ, Long Z *et al.* Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood* 2003; **101**: 2563–2569.
- 77 Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I *et al.* Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 2009; **326**: 818–823.
- 78 Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F *et al.* Transfusion independence and HMG2 activation after gene therapy of human beta-thalassaemia. *Nature* 2010; **467**: 318–322.
- 79 Boulad F, Wang X, Qu J, Taylor C, Ferro L, Karponi G *et al.* Safe mobilization of CD34+ cells in adults with beta-thalassemia and validation of effective globin gene transfer for clinical investigation. *Blood* 2014; **123**: 1483–1486.
- 80 Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C *et al.* Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 2013; **341**: 1233151.
- 81 Biffi A, Montini E, Liorio L, Cesani M, Fumagalli F, Plati T *et al.* Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 2013; **341**: 1233158.
- 82 Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A *et al.* T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011; **3**: 95ra73.
- 83 Naldini L, Blomer U, Gally P, Ory D, Mulligan R, Gage FH *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
- 84 Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci USA* 1996; **93**: 15266–15271.
- 85 Cavalieri S, Cazzaniga S, Geuna M, Magnani Z, Bordignon C, Naldini L *et al.* Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood* 2003; **102**: 497–505.

- 86 Wang GP, Levine BL, Binder GK, Berry CC, Malani N, McGarrity G *et al.* Analysis of lentiviral vector integration in HIV+ study subjects receiving autologous infusions of gene modified CD4+ T cells. *Mol Ther* 2009; **17**: 844–850.
- 87 Ni Y, Sun S, Oparaocha I, Humeau L, Davis B, Cohen R *et al.* Generation of a packaging cell line for prolonged large-scale production of high-titer HIV-1-based lentiviral vector. *J Gene Med* 2005; **7**: 818–834.
- 88 Broussau S, Jabbour N, Lachapelle G, Durocher Y, Tom R, Transfiguracion J *et al.* Inducible packaging cells for large-scale production of lentiviral vectors in serum-free suspension culture. *Mol Ther* 2008; **16**: 500–507.
- 89 Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 1993; **90**: 8392–8396.
- 90 Pham PL, Kamen A, Durocher Y. Large-scale transfection of mammalian cells for the fast production of recombinant protein. *Mol Biotechnol* 2006; **34**: 225–237.
- 91 Toledo JR, Prieto Y, Oramas N, Sanchez O. Polyethylenimine-based transfection method as a simple and effective way to produce recombinant lentiviral vectors. *Appl Biochem Biotechnol* 2009; **157**: 538–544.
- 92 Ansoerge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. *J Gene Med* 2009; **11**: 868–876.
- 93 Witting SR, Li LH, Jasti A, Allen C, Cornetta K, Brady J *et al.* Efficient large volume lentiviral vector production using flow electroporation. *Hum Gene Therapy* 2012; **23**: 243–249.
- 94 Kutner RH, Puthli S, Marino MP, Reiser J. Simplified production and concentration of HIV-1-based lentiviral vectors using HYPERFlask vessels and anion exchange membrane chromatography. *BMC Biotechnol* 2009; **9**: 10.
- 95 Ausubel LJ, Hall C, Sharma A, Shakeley R, Lopez P, Quezada V *et al.* Production of CGMP-Grade Lentiviral Vectors. *Bioprocess Int* 2012; **10**: 32–43.
- 96 Segura MM, Mangion M, Gaillet B, Garnier A. New developments in lentiviral vector design, production and purification. *Expert Opin Biol Ther* 2013; **13**: 987–1011.
- 97 Segura MM, Garnier A, Durocher Y, Ansoerge S, Kamen A. New protocol for lentiviral vector mass production. *Methods Mol Biol* 2010; **614**: 39–52.
- 98 Schweizer M, Merten OW. Large-scale production means for the manufacturing of lentiviral vectors. *Curr Gene Therapy* 2010; **10**: 474–486.
- 99 Kebriaei P, Huls H, Jena B, Munsell M, Jackson R, Lee DA *et al.* Infusing CD19-directed T cells to augment disease control in patients undergoing autologous hematopoietic stem-cell transplantation for advanced B-lymphoid malignancies. *Hum Gene Ther* 2012; **23**: 444–450.
- 100 Huls MH, Figliola MJ, Dawson MJ, Olivares S, Kebriaei P, Shpall EJ *et al.* Clinical application of Sleeping Beauty and artificial antigen presenting cells to genetically modify T cells from peripheral and umbilical cord blood. *J Vis Exp* 2013: e50070.
- 101 Singh H, Figliola MJ, Dawson MJ, Olivares S, Zhang L, Yang G *et al.* Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using Sleeping Beauty system and artificial antigen presenting cells. *PLoS One* 2013; **8**: e64138.
- 102 FDA Process Validation: General Principle and Practices. 2011.
- 103 FDA. Guidance for Industry: CGMP for Phase 1 Investigational Drugs. 2008.
- 104 Khoo HM, Patel N, Carter CS, Murray PR, Read EJ. Sterility testing of cell therapy products: parallel comparison of automated methods with a CFR-compliant method. *Transfusion* 2006; **46**: 2071–2082.
- 105 Hocquet D, Sauguet M, Roussel S, Malugani C, Pouthier F, Morel P *et al.* Validation of an automated blood culture system for sterility testing of cell therapy products. *Cytotherapy* 2014; **16**: 692–698.
- 106 Gee AP, Sumstad D, Stanson J, Watson P, Proctor J, Kadidlo D *et al.* A multicenter comparison study between the Endosafe PTS rapid-release testing system and traditional methods for detecting endotoxin in cell-therapy products. *Cytotherapy* 2008; **10**: 427–435.
- 107 Bouchie A, Allison M, Webb S, DeFrancesco L. Nature Biotechnology's academic spinouts of 2013. *Nat Biotech* 2014; **32**: 229–238.