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Preparing clinical grade Ag-specific T cells for adoptive immunotherapy trials

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Abstract

The production of clinical-grade T cells for adoptive immunotherapy has evolved from the *ex vivo* numerical expansion of tumor-infiltrating lymphocytes to sophisticated bioengineering processes often requiring cell selection, genetic modification and other extensive tissue culture manipulations, to produce desired cells with improved therapeutic potential. Advancements in understanding the biology of lymphocyte signaling, activation, homing and sustained *in vivo* proliferative potential have redefined the strategies used to produce T cells suitable for clinical investigation. When combined with new technical methods in cell processing and culturing, the therapeutic potential of T cells manufactured in academic centers has improved dramatically. Paralleling these technical achievements in cell manufacturing is the development of broadly applied regulatory standards that define the requirements for the clinical implementation of cell products with ever-increasing complexity. In concert with academic facilities operating in compliance with current good manufacturing practice, the prescribing physician can now infuse T cells with a highly selected or endowed phenotype that has been uniformly manufactured according to standard operating procedures and that meets federal guidelines for quality of investigational cell products. In this review we address salient issues related to the technical, immunologic, practical and regulatory aspects of manufacturing these advanced T-cell products for clinical use.

Keywords

T-cell manufacturing; immunotherapy; good manufacturing practice; gene therapy

Introduction

The role of T cells in preventing and curing advanced malignancies is unequivocal. This is perhaps best exemplified in the field of allogeneic hematopoietic stem-cell transplantation (HSCT), where engrafted donor-derived T cells are capable of curing hematologic malignancies, commonly known as the graft vs. tumor (GvT) effect. Extrapolating from this experience, investigators have sought to adoptively transfer T cells with defined specificity to augment allogeneic HSCT and possibly avoid the antithetical clinical outcome of GvHD. Understanding what (e.g. CD8⁺ and/or CD4⁺, memory vs. effector, T_{reg}) and whose (e.g. allogeneic vs. autologous) T cells to infuse, and when (e.g. during lymphopenia) and why (e.g.

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to treat bulky disease vs. minimal residual disease) to infuse, has for years preoccupied the field of adoptive immunotherapy. The advent of clinically acceptable methods for cell fractionation, genetic manipulation (e.g., introducing recombinant T-cell receptors) and *ex vivo* expansion [unbreached cell processing and culture systems, addition of exogenous good manufacturing practice (cGMP)-compliant cytokines] has led to a burgeoning of the cell therapeutics field, in which extensive *ex vivo* manipulation strategies have been evaluated in an effort to recapitulate the allogeneic GvT-effect while minimizing undesirable non-target immune reactivity. The state-of-the-art has rapidly evolved to the point where academic investigators can design clinical trials based upon selection or generation of T cells from desired subpopulations and chose antigen (Ag)-specific or antigen-independent expansion strategies to maintain target specificity and *in vivo* persistence with little to no deleterious reactivity to healthy tissue. In this review, we describe some of the critical parameters associated with the manufacturing of T cells with these desirable attributes.

Identifying/selecting a desired T-cell population for adoptive immunotherapy

The production of a clinical T-cell product begins with the selection of a cell type upon which to apply selection, propagation and/or one or more gene engineering processes. One approach has been to isolate T cells with endogenous $\alpha\beta$ T-cell receptors (TCR) that are *a priori* recognized to be specific for disease-related Ag. Examples include tumor-infiltrating lymphocytes (TIL) isolated from excised solid tumors, draining lymph nodes, and the peripheral blood of patients, sometimes subsequent to tumor Ag vaccination to boost precursor frequency [1–5]. Using a strategy to massively expand TIL while retaining Ag specificity, Rosenberg and colleagues at the National Cancer Institute (NCI, Bethesda, MD, USA) have undertaken extensive investigations to demonstrate objective responses in melanoma patients by treating them with *ex vivo*-expanded tumor-specific T cells in conjunction with a lymphodepleting conditioning regimen [6–8]. The ability to propagate T cells efficiently from small numbers of recovered cells provides the opportunity to manufacture populations of T cells to $>10^{10}$ cells/dose. While melanoma-specific clones were originally infused to provide a homogeneous population from which to study, these TIL-derived clones failed to exhibit anti-tumor responses, partly because of the disappearance of the infused T cells despite the use of lymphodepleting conditioning therapy [9]. In contrast, adoptively transferred T-cell oligoclonal lines similarly infused after chemotherapy were associated with tumor regression [10]. Presumably this was because of the outgrowth of subsets of cells from the infused lines reflecting *in vivo* selection of T cells capable of recognizing tumor Ag [11]. This therapeutic success could be attributed further to the persistence of both CD4 ‘helper’ and CD8 ‘cytolytic’ tumor-specific cells present in the T-cell lines coupled with the use of lymphodepleting chemotherapy to provide ‘space’ for the homeostatic proliferation of the infused T cells.

Similarly, the isolation and expansion of virus-specific T cells from CMV or EBV seropositive individuals to treat viremia or lymphoproliferative/malignant disorders in immunocompromised patients has shown great promise [12–18]. These studies have demonstrated that the *in vivo* survival of CD8⁺ T-effector cells is improved by the presence of CD4⁺ helper cells. However, it is probable that long-term engraftment of virus-specific cells in these studies may be because of the persistence of viral Ag as a constant ‘driver’ of virus-specific cellular proliferation [19]. Attempts to replace the requirement for CD4-mediated ‘help’ and endogenous production of IL-2 by administering the recombinant human IL-2 (rhIL-2) cytokine has engendered considerable discussion related to the dose and scheduling of rhIL-2 to sustain the biologic activity of infused CD8⁺ cells (Table 1) [20,21]. For example, Yee *et al.* [22] have performed a randomized trial demonstrating a possible role for low-dose rhIL-2 infusions to reduce systemic toxicity. As an alternative approach to reducing systemic toxicity, other laboratories are investigating the effects of delivering cytokine to the neoplasm using an immunocytokine fusion protein that targets IL-2 to the tumor microenvironment via

a tumor Ag-specific monoclonal antibody binding domain [23]. The specific role for, and dosing of, other immunomodulatory cytokines (especially IL-7, IL-15 and IL-21) to support the persistence and anti-tumor effect of infused genetically modified and unmodified T cells is beginning to be the subject of clinical investigation. Such studies will need to yield consistent objective results regarding the role for exogenous cytokines before general guidelines can be established.

Another critical parameter to improving *in vivo* T-cell survival is the selection of a cell substrate from which to produce populations for infusion. T cells can be divided into several subsets with varying biologic function based on the co-ordinated expression of cell-surface markers such as L-selectin (CD62L), chemokine receptors related to cell homing (CCR7) and various isoforms of the protein tyrosine phosphatase CD45 (CD45RA, CD45RO). Several theories have emerged describing the transition of T cells from naive to memory, a process that may be followed through serial flow cytometric analyzes of the cell-surface expression of the above-mentioned Ag. Using these principles, central memory (CM) CD62L^{hi}/CCR7⁺ T cells have been shown to home to lymph nodes while effector memory (EM) CD62L^{lo}/CCR7⁻ T cells migrate to gut and other lymphoid organs. Purification of T cells expressing each specific phenotype has been performed to determine which subset provides the best long-term engraftment and continuous production of effector cells with potent antiviral and/or anti-tumor activity. Wherry *et al.* [24] have dissected the progression of naive cells to effector cells and then to either the T_{CM} or T_{EM} counterpart using a murine LCMV viral infection model. Their data indicate that the CD62L^{hi}/CCR7⁺ T_{CM} cells provide longer lasting immunity than the T_{EM} cells. Other studies have shown that this principle holds true for tumor cell models [25]. In an evaluation of DC vaccination strategies, Liu *et al.* [26] have demonstrated that *in vitro*-activated and expanded CD62L-expressing cells (isolated from the draining lymph nodes of mice vaccinated with tumor-pulsed DC) were an important component in curing pre-established metastatic tumors in mice. Zhang *et al.* [27,28] have expanded this concept by demonstrating the existence of a population of murine T cells, defined in part as CD62L^{hi}/CD8⁺, that have properties consistent with being a T-memory 'stem' cell that gives rise to T_{CM} and have been shown to be required for sustaining disease in a model of chronic GvHD. Using a non-human primate model of CMV infection, Berger *et al.* [29] have shown that *a priori* adoptive transfer of genetically marked MACAQUE CD62L⁺ T_{CM} clones that had been rapidly expanded *ex vivo*, persisted for longer *in vivo* compared with differentiated (CD62L^{neg}) T_{EM} clones. In aggregate, these data raise the possibility that *ex vivo* sorting/selection for T_{CM} (e.g. CD62L^{hi}/CCR7⁺) cells prior to adoptive transfer and/or gene transfer could provide a source of cells with improved *in vivo* persistence and therapeutic efficacy.

Engineering T-cell specificity

As an alternative or complement to selecting T cells with endogenous tumor specificity, many groups have reported on efforts to 'engineer' tumor specificity into T cells by gene transfer of $\alpha\beta$ TCR or a single-chain chimeric Ag receptor (CAR) with specificity for tumor or pathogen [30–37]. Using this approach, Ag specificity can be engineered/introduced into T cells without the need for prolonged Ag-driven selection. However, the transfer of $\alpha\beta$ TCR pairs appears to have limitations relative to the CAR strategy. For example, many tumors are known to avoid an immune response by down-regulating MHC expression, thus preventing MHC-based Ag recognition altogether [38]. Furthermore, Ag recognition using a transgenic $\alpha\beta$ TCR pair requires knowledge of recipient HLA (specific MHC class I or class II allele) so that the cloned TCR can maintain recognition of cognate Ag presented by MHC protein on the tumor or infected cell. Finally, the specificity of a transferred $\alpha\beta$ pair may be altered through unwelcome pairing with the endogenous $\alpha\beta$ TCR chains in the recipient cell, a situation that could lead to undesired thymic-unselected auto-reactive receptor pairs. However, recently investigators have provided methods to limit undesired mispairing between endogenous and introduced

$\alpha\beta$ TCR chains, which include fusing TCR α and β chains to CD3- ζ , including cysteine residues to facilitate inter-chain disulfide bond formation, and swapping human TCR constant domains with murine counterparts [39–42].

The use of CAR avoids many of the pitfalls of $\alpha\beta$ TCR pairs by combining a single-chain Ab recognition domain (or Ag-recognition domain) with a transmembrane motif and one or more intracellular signaling domains. T cells with enforced expression of CAR are currently being evaluated in clinical adoptive immunotherapy trials for hematologic malignancies and solid tumors, targeting a defined cell-surface tumor-specific or lineage-specific Ag [43]. For example, CAR⁺ T cells specific for lymphoma, ovarian cancer, neuroblastoma, renal cell cancer and HIV are currently, or have been, in early phase clinical trials [44–50]. While it is well established that the CD3- ζ chain alone can transduce a T-cell signal to activate cytotoxicity [51], persistence of adoptively transferred CAR⁺ T cells signaling solely through CD3- ζ have generally been short lived *in vivo*, compromising tumor clearance. Recent data have demonstrated that the addition of various intracellular co-stimulatory domains can enhance T-cell activation, proliferation and perhaps persistence of these cells after adoptive transfer [52, 53]. Second-generation CAR constructs are now being developed that include the intracellular signaling components of CD28, 4-1BB and other co-stimulatory models in an attempt to improve the activity of adoptively transferred CAR-expressing T cells [54–58]. However, a careful balance must be struck to avoid over-stimulation of T cells leading to apoptosis or potentially an undesired phenotype capable of autonomous Ag-independent replication. The latter possibility (transformation) has prompted the inclusion of suicide genes in expression vectors encoding the CAR constructs as a safety feature to provide conditional ablation of the infused genetically modified T cells in the event of uncontrolled cell proliferation or other serious adverse events. While suicide genes, such as HSV-1-derived thymidine kinase (TK), have been shown to eliminate the TK⁺ T cells upon exposure to the pro-drug ganciclovir, a viral-derived TK may result in the elimination of cells by the host immune system [59,60]. Despite the potential immunogenicity of HSV-1 TK, investigators in Europe have infused donor-derived TK⁺ T cells after allogeneic HSCT and commented that the anti-TK immune response did not lead to the loss of the GvT effect [61,62]. As an alternative to TK, investigators have developed non-immunogenic alternatives, including over-expression of human-derived selectively dimerizable caspase-9 and thymidylate kinase [63,64]. Thus the inclusion of intracellular signaling domains with or without conditional suicide genes requires careful consideration before clinical applications are undertaken.

A choice must also be made on how to introduce one or more transgenes into T cells. Initially, murine leukemia virus gamma-retroviral vectors were used efficiently to introduce transgenic receptors into T cells. However, the production and testing of retroviral producer cell lines and cGMP batches of virus remains expensive and time consuming, and can prevent efficient iterative improvements in vector or transgene design. Furthermore, the discovery of transgene-containing lymphoid malignancies in children treated with retrovirally transduced hematopoietic stem cells (HSC) has discouraged some investigators from pursuing this vector system for adoptive T-cell trials [65]. Our experience using naked plasmid DNA and electroporation has proven to be efficacious in terms of a safe, reproducible, economically feasible and flexible platform for construct evaluation, but may be limited by the lengthy time for selection of cells containing stable genomic transgene integrants. A compromise may be the use of DNA transposon/transposase elements, e.g. from *Sleeping Beauty*, which have the ability to transfect T cells efficiently, do not show apparent preferential integration at loci associated with disease induction, and can be manufactured according to cGMP standards [66–70].

Cell processing

Peripheral blood is a readily available source of large numbers of T cells that can be accessed by apheresis or venipuncture and enriched in a mononuclear cell fraction. Traditional mononuclear cell fractionation methods such as Ficoll or Percoll gradients have given way to new processing strategies using closed systems, such as the semi-automated CytoMate™ (Baxter Biotechnologies, Deerfield, IL, USA) and Elutra™ (Gambro BCT, Lake-wood, CO, USA) cell-processing devices. The CytoMate fractionates cells based on size using a spinning membrane filter, while the Elutra uses the principle of counter-flow centrifugal elutriation to separate cell populations by size and density. These devices are configured to allow aseptic connections with blood bags and thus reduce the likelihood of contamination of the products. The CytoMate device has been used extensively for washing clinical cell preparations, and isolated mononuclear cell preparations exhibit high viability (>90%) and can be cultured *in vitro* to generate T cells with efficiencies comparable to Ficoll-prepared cells [71]. Monocyte recoveries from the Elutra also exceed 90% with purities of >80% and cell preparations enriched in this manner have been used *in vitro* successfully to generate DC for clinical use (see below) [72]. The Elutra is currently being used in our laboratories to enrich monocytes as a source of antigen presenting cells (APC) for selecting Ag-specific T cells and to wash *ex vivo*-expanded T cells prior to formulation for infusion.

Ex vivo cell-processing technologies can also be used to isolate T cells expressing desired cell-surface markers or purge tumor cells expressing tumor-specific Ag. Typically this is achieved using MAb to direct specificity and (most commonly) with magnetic beads or paramagnetic particles as a capture strategy. Paramagnetic clinical devices, including the Isolex 300i (Baxter) and Clini-MACS® (Miltenyi Biotec GmbH Bergisch Gladbach Germany), have been tested extensively and demonstrate sufficient yields and recoveries (typically around 60–70%) with high cell purity (>95%) to warrant their use in depleting or enriching cells based on cell-surface Ag expression [73–77]. These closed systems are compliant with regulatory requirements for cell processing. The Isolex device is approved by the FDA for clinical use (in the USA), but only for autologous CD34⁺ HSC selection for oncology indications. The CliniMACS has received CE Mark certification in Europe, where it is available for similar clinical use. Both devices, however, are available for investigational new drug (IND)-sponsored clinical research investigations through a manufacturer's letter of authorization to investigational device exemptions (IDE) and/or device master files.

Despite this bioengineering infrastructure, the application of selection/purging is typically constrained to those procedures for which clinical-grade Ab is available. Also, selection/depletion of cell populations based on more than one surface marker requires sequential product processing and is subject to low cumulative yield, especially when the Ag of interest is expressed at low densities on the cell surface. To augment or replace magnetic bead separation technologies, some centers, such as ours, have invested in high-speed FACS within their GMP-facilities [78]. This approach has been shown (in limited circumstances) to result in the isolation of homogeneous cell products with desired phenotypes. However, FACS has the same requirements for cGMP-grade reagents as magnetic bead-based procedures, is subject to the failure rate of a complicated electronic device, requires dedicated staffing, and has safety issues related to the generation of aerosols containing potentially infective particles or where product contamination can occur [79]. This latter problem (aerosol generation) can be addressed using class 100 enclosures for the sorting chamber and, more recently, by the advent of non-aerosol-generating microfluidic sorting devices (Gigasort™; Cytonome, Boston, MA, USA) [80–82]. One clinical application currently in development is the sorting of hematopoietic, endothelial, and mesenchymal stromal cells based on aldehyde dehydrogenase activity [83–86]. Many of the issues related to the regulatory filing associated with utilizing these devices is in the hands of the device manufacturers, and thus this area of specialization is likely to

requiring extensive corporate collaboration. Given the complexity of operation, limitations in reagent availability, cost of equipment, and limited regulatory support available, the use of cell sorting as a strategy to perform multi-parameter isolation of substrate cell populations is not yet ready for extensive clinical utilization, but is a critical area for development.

Methods to propagate T cells

There are numerous approaches to expanding clinical-grade T cells, and the choice of method depends on the construct and desired end-product. Several of these methods are schematically represented in Figure 1. Efficient and sustained *in vitro* expansion of human T cells generally requires activation through the endogenous TCR complex or cross-linking chimeric CD3- ζ , with a resulting signaling cascade that includes co-ordinated co-stimulatory signals, such as those mediated through the CD28 molecule either in its endogenous or chimeric state. In addition, the continued survival and maximum expansion of T cells benefit from signals provided through other co-stimulatory receptors, such as 4-1BB, CD40 and OX40. A variety of methods have been reported to achieve Ag-specific expansion of T cells. Our laboratories have extensively utilized the rapid expansion method (REM) of Riddell *et. al.* [87], which induces T-cell activation through the combined action of a MAb to the TCR CD3 ϵ domain (Orthoclone OKT-3; RW Johnson, Raritan, NJ, USA), provision of exogenous rhIL-2 (Proleukin, Chiron Corp., Alameda, CA, USA) and feeder cells in the form of γ -irradiated allogeneic PBMC and a γ -irradiated allogeneic EBV-transformed B-lymphoid cell line (LCL). Ligation of CD3 with the OKT3 Ab is sufficient to provide the first signal, and the interaction of CD28 with CD80 (and other membrane-bound co-stimulatory cells with their cognate ligands) on the γ -irradiated PBMC/LCL feeders is sufficient to provide the requisite co-stimulatory signals. Cultures in our laboratories are maintained in 10–50 IU/mL rhIL-2 and fed by demi-depletion as cell density increases and nutrients are consumed. Typically, cells become quiescent about 14 days after OKT3 activation, at which time the stimulation is repeated. This method allows for extensive bulk expansion of T cells and has been shown to support the expansion of individual clones of T cells to doses of more than 10^{10} cells for clinical use. However, prolonged culture of T cells with this method obtained from healthy volunteer donors typically results in the production of terminally differentiated CD8⁺ effector cells [88]. These cells, while possessing strong cytolytic activity, do not appear to persist for long periods *in vivo* and thus may not provide long-lasting protection against tumor recurrence. Expansion of CD4⁺ cells can be achieved using this system through prospective enrichment of CD4⁺ cells or depletion of CD8⁺ cells from the original source of T cells. The REP T-cell expansion method has been utilized extensively for phase I/II adoptive immunotherapy studies in lymphoma, glioma, neuroblastoma and viral disease indications. OKT3 and rhIL-2 are readily available as clinical-grade products, but the provision of allogeneic PBMC requires the development of institutional protocols and infrastructure for identifying and consenting donors, performing apheresis and processing and testing samples for suitability in clinical applications. Clinical-grade allogeneic LCL have been manufactured and are available from a number of centers (such as the GMP facility at MDACC and the Center for Biomedicine and Genetics at the City of Hope).

A limitation of this REP strategy is that acquiring and maintaining the feeder-cell resources is expensive and may be impractical for larger trials where the production of large numbers of patient-specific product is required. Attempts have been made to replace PBMC/LCL feeders with artificial APC (aAPC), with enforced expression of stimulatory molecules such as anti-CD3 and anti-CD28 MAb, Ag and/or co-stimulatory ligands [89–93]. These aAPC have been shown to activate T cells and improve the duration of proliferation of cells expanded in this manner without having to rely on primary isolates of allogeneic PBMC. To date these methods are considered promising, and these cells are being manufactured as master cell banks according to cGMP for phase I/II trials.

Alternative methods for T-cell activation that provide both a TCR signal and requisite co-stimulatory signals independent of co-stimulatory cells have been described. June and colleagues [94] have demonstrated the use of Ab to CD3 and CD28 immobilized on magnetic beads to achieve and sustain high levels of numeric expansion of T cells from lymph nodes and peripheral blood. Using this method, Levine and others have demonstrated up to 10 000-fold expansion of T cells from HIV⁺ patients with recovery of a diverse TCR V β repertoire and functional competence (cytokine secretion) [95–97]. Similarly, CD3/ CD28 bead expansion of T cells has been utilized to propagate autologous T-cell populations for treating CLL, with a concomitant 400-fold depletion of malignant B cells during cultivation in a disposable 20-liter bioreactor [4]. *Ex vivo*-expanded T cells have also been used for donor leukocyte infusions (DLI) in conjunction with allogeneic HSCT for hematologic malignancies. Infusions of 10⁶–10⁸ *ex vivo*-expanded T cells/kg were well tolerated, not associated with severe GvHD, and may contribute to durable remissions [98,99].

The use of Ag-pulsed DC has also been shown to elicit rapid and sustained Ag-specific T-cell proliferation [100–103]. DC express high levels of T-cell co-stimulatory molecules, such as CD40, CD80, CD86, HLA class I and II and 4-1BBL, and thus initiate the full activation cascade required for T_H and cytotoxic effector-cell expansion. A variety of methods has been explored to load DC with a source of tumor Ag (e.g. tumor cell lysates, tumor-specific RNA and antigenic peptides). Most investigations have utilized magnetic bead selection to isolate clinical-grade CD14⁺ monocytes from peripheral blood, followed by induction of *in vitro* differentiation into DC (e.g. using GM-CSF and IL-4), Ag loading and finally, activation with TNF- α . Numerous clinically acceptable methods have been developed for this manufacturing process, including completely closed systems for generating the professional APC products [72,104–108].

Media and cytokines

One of the most important factors in the *ex vivo*-expansion of T cells is selection of the appropriate culture media. Current trends in formulation have allowed for a transition from media containing FBS and other ruminant animal material (e.g. insulin and transferrin) to serum-free or chemically defined media. Not all media perform equally, and a screening process is often utilized to determine which media is optimal for the culture of T cells for a particular application [96,109–112]. Quite often the criteria used to screen media include proliferation and viability of cells. These parameters, while important, can not alone determine the utility of a particular media formulation. For example, the combination of nutrients, lipids, prostaglandins, trace elements and amino acid composition can significantly affect culture outcomes such as the ability of APC to present Ag [113,114], the T cells to recognize, proliferate and secrete cytokines in response to the Ag [115], or the final phenotype of cells resulting from culture in the media [116]. Two of the more frequently used media for *ex vivo* culture of T cells include X-VIVO15 and AIM-V [94,96,109,111,116]. Both of these media support 10³–10⁴-fold expansion of T cells, including both CD4⁺ and CD8⁺ cells with functional attributes (cell killing and T_H1 cytokine production). However, the ability of these serum-free media to support maximal proliferative and functional potential of T cells still requires the addition of exogenous cytokines. Among the cytokines studied for their effect on cultured T cells are IL-2, IL-7, IL-15, IL-12 and IL-21. Dosing of rhIL-2 in support of T cells *in vitro* is varied from low-dose IL-2 at 10 U/mL to 6000 U/mL. Recently, exogenous rhIL-2 and rhIL-15 have been combined in our laboratories [89], as IL-2 and IL-15 share a common β chain (CD122) and γ_c chain (CD132) and induce a growth-promoting signal through both Jak1 and Jak3 pathways, but the unique IL-15 α receptor also binds TRAF2, whose sequestration leads to inhibition of activation-induced cell death [117,118]. Furthermore, IL-15 has been shown to prevent Fas-induced apoptosis and overcome tolerance of tumor-specific CD8⁺ T cells [119–121]. However, the provision of cytokines suitable for clinical cell manufacturing,

particularly for advanced-phase trials, is limited to those cytokines already available as clinical entities. Only a few companies are currently producing cytokines, especially members of the common γ -chain family, with implications for T-cell immunobiology (with or without clinical indication for use as a parenteral drug) under cGMP regulations. Examples include Gentaur (Brussels, Belgium), Strathmann Biotech (Hamburg, Germany), Cell-Genix (Freiburg, Germany) and ZymoGenetics (Seattle, WA, USA).

Determining cell dose

The minimal dose of T cells to be adoptively transferred to achieve a therapeutic effect is not known and is probably dependent on (a) manufacturing conditions (e.g. avoiding extensive propagation that might lead to replicative senescence), (b) phenotype (e.g. terminally differentiated effector cells might not persist *in vivo* as long as T_{CM}), (c) recipient condition (e.g. infused T cells can numerically expand in a lymphopenic environment), (d) tumor site (e.g. the dose of systemically administered T cells will be higher than loco-regional administration, such as within a resection cavity of a brain tumor), (e) ability of infused T cells to recycle killing (avoid one-hit kinetics) and (f) relative densities of expressed immunoreceptor and tumor Ag. A therapeutic effect has been described for infusion of Ag-specific T cells that vary in dosing: c. $0.2\text{--}10^5$ cells/kg to 10^5 /kg (infusion of CMV-specific T-cell lines) [122], a single infusion of $5\text{--}10^6\text{--}10^8$ cells/m² (multivirus-reactive T cell line) [123], $4\text{--}10^7\text{--}3\text{--}10^8$ /m² over 1 or 2 infusions (EBV-specific T cells) [124], $0.11\text{--}13.1\text{--}10^8$ cells/infusion for at least three infusions (melanoma-specific T cells) [133] and $2.3\text{--}10^{10}\text{--}13.7\text{--}10^{10}$ (melanoma-specific T cells) [10]. The dosing of T cells to achieve a desired clinical response through endogenous (or introduced) $\alpha\beta$ TCR is likely to be different than T cells that are activated through an introduced CAR. Further complicating the judgment of how many cells to infuse is that T cells expressing immunogenic transgenes may be eradicated because of immune-mediated mechanisms, particularly in the absence of immunosuppression (lymphodepleting chemotherapy) or HSCT conditioning regimens [61].

Regulatory environment

In the USA, *ex vivo*-manipulated cell products for infusion (that are more than minimally manipulated) must undergo review as an IND with the FDA. This requires successful filing of an IND application, institutional review board (IRB) approval of the protocol and consent (or consents if donor-derived allogeneic cells are used), and is governed by (but not limited to) the standards set out in the Code of Federal Regulations (CFR) sections 21CFR 210, 211 (Good Manufacturing Practices or GMP), 21 CFR 1270 Human Tissue Intended for Transplantation and 21 CFR 1271 Human Cells, Tissues, and Cellular and Tissue-Based Products (collectively referred to as Good Tissue Practices or GTP). GMP regulations dictate the requirement for a quality-systems approach to T-cell manufacturing that includes control over manufacturing, personnel, facilities, raw materials compounding, and final product release, as well as a complete documentation system for production, testing and product release. GTP add to the requirements for facility registration (when producing cell- or tissue-based products that are not part of an IND filing), mandate extensive screening and testing of donors of cells that are used to make products, and require manufacturers to provide protection of products during processing to prevent contamination or other adulteration. When gene transfer is proposed as part of the manufacturing process, specific additional approval is required from an institutional biosafety committee (IBC), which reviews compliance with reporting requirements defined in the FDA's 'Points To Consider in the Design and Submission of Protocols For The Transfer of Recombinant DNA Molecules Into One or More Human Research Participants', also known as 'Appendix M', and by the National Institutes of Health Office of Biotechnology Activities (NIH-OBA) and NIH Recombinant DNA Advisory Committee (RAC) [125,126].

Production of clinical-grade cell products usually occurs in dedicated facilities that meet or exceed FDA requirements for environmental quality, which typically includes (a) class 10 000 clean rooms equipped with class 100 biosafety cabinets where open cell culture manipulations are performed, (b) unidirectional processes for material and personnel to flow from clean to 'gray' or downstream areas, (c) constant surface and air monitoring for bioburden, and (d) documented effective cleaning and room change-over procedures to prevent cross-contamination. Dedicated areas for gowning, quarantined and released materials, product processing and storage, waste disposal and facility services (process gasses, autoclaves and dishwashers) are also commonly provided. Access to all manufacturing, equipment and materials is restricted at all times to qualified and authorized individuals. Staffing of these facilities usually includes facility engineers, equipment specialists (to manage complicated cell processing devices, bioreactors, cell sorters), quality control and quality assurance officers, and specially trained janitorial personnel. A diagram of a prototypic facility for manufacture of clinical-grade T cells in compliance for phase I/II trials is provided in Figure 2.

Once *ex vivo*-manufactured T cells have been produced, they must meet release criteria designed to ensure the identity, purity, number, viability, safety and sometimes potency of the product. T-cell identity, purity and viability may be performed by flow cytometric analysis of cell-surface markers. Safety testing includes standard parameters such as low to non-detectable levels of endotoxin and freedom from mycoplasma, bacterial and fungal contamination. Some sterility tests may be performed post-infusion if freshly manufactured cells are to be immediately infused, but tests for gross contamination (e.g. Gram and KOH staining) are usually required prior to infusion in these cases. Additional safety tests to ensure the products are not transformed are advisable. Stability assays should be performed if the product is to be administered after storage. Potency is often a target-based stimulation or cell elimination assay, such as cytokine production or tumor cell lysis, in response to stimulation with appropriate Ag. Specialized testing, such as flow cytometry and/ or Western blotting, is typically needed to document the presence of introduced transgenes, such as a CAR. When the transgene is a suicide gene, assays are needed to demonstrate conditional ablation of the T cells. Examples of the release criteria used for T cells created for clinical trials performed under a current IND for CAR-redirectioned T cells are listed in Table 2. Product release, as described above, is tested by a dedicated quality control group that is functionally and administratively independent of the manufacturing group (and principal investigator), to avoid conflict of interest. Some testing may also be performed by contract testing agencies. A typical organizational structure that ensures freedom from conflicts of interest is shown in Figure 3. Finally, the released products must be secured and stored to prevent degradation and contamination and maintain chain of custody. These procedures are governed by labeling and stability guidance documents provided by the FDA.

Challenges

With sufficient notice, manufacturing facilities in academic facilities operating in compliance with cGMP for phase I/II trials can reliably produce Ag-specific T cells in large numbers suitable for infusion. By itself, this is a tremendous achievement and has resulted in clinical trials establishing that adoptive transfer of these cells can result in destruction of tumor and elimination of pathogens in clinical trials. Furthermore, this T-cell propagation technology has been coupled with gene transfer and early-phase trials have established the proof-of-principal that genetically modified cells can be safely infused and, in some cases, result in an anti-tumor effect.

However there are a number of hurdles to be overcome before T-cell therapy can enter mainstream clinical practice. One challenge is to shorten the period of *ex vivo* propagation so that T cells can be infused with the ability to avoid replication senescence and potential to home

to secondary lymphoid organs. These minimally manipulated T cells can then be infused ‘on demand’ without the cost of maintaining cells for a long time in tissue culture in compliance with cGMP.

A second challenge is to develop expression vectors with integration targeted to favored sites to avoid adverse events related to insertional mutagenesis. The excellent safety record of long-term (greater than 15 years) gene-marked T cells [127] and more than 100 infusions of allogeneic T cells transduced to express TK [128] are encouraging, and to date there have been no reported adverse events attributed to gene transfer in T cells. Thus genotoxicity resulting from deleterious integration of transgene and/or introduced promoter does not currently appear to be a major problem for adoptive transfer human T cells. However, as trials are undertaken to infuse populations of T cells that include genetically modified memory T cells, with associated stem-cell-like qualities of self-renewal, the potential to give rise to other T-cell subsets and long-term survival, it will be important for the field of adoptive immunotherapy to avoid the problems associated with HSC gene therapy and the potential for leukemia caused by gamma-retroviral vector integration. Here the application of T-cell therapy is helped by the development of transposon/transposase DNA vectors and self-inactivating lentiviral vectors to transduce quiescent T cells, as these vectors exhibit preferred integration that may avoid the genotoxicity associated with gamma-retroviruses that preferentially integrate near transcription start sites [129,130].

A third challenge is developing the manufacturing and clinical infrastructure to undertake multi-center clinical adoptive immunotherapy trials that will be needed to establish and validate efficacy of genetically modified T cells. Complicating this goal is implementing the legal contracts at each infusion site to indemnify the party that developed the expression vector and manufactured the cells.

A fourth challenge is obtaining relief for the high-cost of infusing genetically modified T cells. At present this technology is not supported by biomedical companies and the financial burden for implementation falls to non-profit academic facilities. As most are using viral vectors to transduce T cells, it is difficult for investigators and institutions to afford and justify producing more than one viral vector and to make the recursive changes needed to go from bench to bedside in a logic series of early phase trials to achieve the therapeutic potential of genetically modified T cells.

Just as there has been intensive research on development of tumor-specific T cells, so there needs to be further development of the methods and infrastructure to manufacture potent cell-based biologics for widespread application and testing. The pay-off for these investments is large, as combining cell therapy with gene therapy has the potential to cure tumors refractory to conventional therapies.

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Figure 1.

Schematic of relative propagation strategies to expand T cells numerically through TCR complex or CAR (shown as second generation capable of Ag-dependent signaling through chimeric CD3- ζ and CD28). (A) REP for robust and rapid Ag-independent T-cell proliferation by cross-linking CD3 ϵ using OKT3 (and also if needed cross-linking CD28) bound to PBMC or aAPC. (B) Selective stimulation of (CD8⁺) T cells for Ag-dependent proliferation through TCR recognizing Ag in the context of HLA class I on APC or aAPC. A co-stimulatory signal initiated through B7 molecules (or anti-CD28) through CD28 is shown. (C) REP of genetically modified T cells co-expressing dominant selection marker (e.g. *Escherichia coli* hygromycin phosphotransferase) for Ag-independent proliferation by cross-linking CD3 (and CD28 if necessary) resulting in selection for outgrowth of CAR⁺ T cells in the presence of cytotoxic concentrations of drug, e.g. hygromycin B. Incomplete CAR expression in stable transfectants may occur if CAR and drug-selection transgenes are under the control of separate promoters. (D) Selective stimulation of genetically modified T cells, in the presence of IL-15, for Ag-dependent proliferation through introduced CAR recognizing cell-surface Ag (e.g. CD19) independent of HLA class I on aAPC. The aAPC are typically modified to enforce expression of desired co-stimulatory molecules, e.g. 4-1BBL, CD80/86. (Figure not to scale and showing selected disulfide bonds).

Figure 2.

Schematic of an example of the physical infrastructure needed to manufacture clinical-grade T cells for phase I/II trials in compliance with current GMP. Not shown are the environmental control systems. The physical plant is staffed by dedicated and highly trained personnel following standard operating procedures (SOP) that conform to cGMP guidelines (not to scale).

Figure 3. Outline of single-institution reporting structure and requirements for manufacture and release of clinical-grade genetically modified T cells in compliance with cGMP for phase I/II trials.

Table 1

General dosing of rhIL-2 in conjunction with adoptive immunotherapy clinical trials

Dosing after lymphocyte infusion	Dosing	Route	Duration	References
0.72×10^6 IU/kg	Every 8 h	Intravenous (bolus) over 15 min	As tolerated for 4–15 doses	131,132
0.72×10^6 IU/kg	Every 8 h	Intravenous (bolus) over 15 min	As tolerated for 2–14 doses	31
0.72×10^6 IU/kg	Every 8 h	Intravenous (bolus) over 15 min	As tolerated up to 12 doses	10
0.75×10^6 IU/kg	Every 8 h	Intravenous (bolus) over 15 min	As tolerated up to 12 doses	9
0.125×10^6 IU/kg	Daily	Subcutaneous	12 days	9
3×10^6 IU	Daily	Subcutaneous	6 days	133
0.25×10^6 , 0.50×10^6 , 1×10^6 IU/m ²	Twice daily	Subcutaneous	14 days	22
1.75×10^6 IU/m ²	Daily	Subcutaneous	14 days	1
10×10^6 IU	3 times weekly	Subcutaneous	639 doses	134

Table 2

Sample release criteria for phase I/II trials of genetically modified cytolytic CD19-specific CAR⁺ T cells co-expressing HSV-1-derived TK suicide gene and propagated by REP

	Test	Sample release criteria	Testing method
Sterility testing	Bacteria and fungi	Negative at 14 days (bacteria) and 28 days (fungal)	USP Bacterial/ fungal culture
	Bacteria and fungi	Negative at time of infusion	Gram and KOH stain
	Endotoxin	Endotoxin level B5 EU/kg patient body weight/h of T-cell infusion	Chromogenic limulus amoebocyte lysate (LAL)
Expression of suicide gene	Mycoplasma	Negative	PCR
	Sensitivity to 5 μ M ganciclovir	Cell growth $\leq$ 10% of positive control	Compared with same T cells stimulated without ganciclovir
Screen for unwanted autonomous cell growth	Dependence on IL-2 for growth	Cell growth $\leq$ 10% of positive control	Compared with same T cells stimulated with IL-2
Phenotype [*]	T-cell-surface phenotype	$\geq$ 90% CD3 ⁺ , $\geq$ 10% CAR ⁺ , <5% CD19 ⁺	Flow cytometric evaluation prior to washing/concentrating
Potency [†]	Western blot	Expected band	Western blot
	CD19-specific cytolytic activity	$\geq$ 30% specific lysis at E:T ratio of 50:1 against Ag ⁺ line	4-h chromium-release assay
Viability	Viability of clinical preparation	$\geq$ 85%	Trypan blue or propidium iodine exclusion test
Identity	HLA typing	PCR	Matches input cells

* Two independent measurements of the introduced transgene.

[†] A potency assay may be part of in-process testing and not release testing for phase I/II trials.