Published in final edited form as:
Cytotherapy. 2012 August ; 14(7): 775–790. doi:10.3109/14653249.2012.694420.

Current translational and clinical practices in hematopoietic cell and gene therapy

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Abstract

Clinical trials over the last 15 years have demonstrated that cell and gene therapy for cancer, monogenic and infectious disease is feasible and can lead to long-term benefit for patients (1). These trials however have been limited to proof of principle and were conducted on modest numbers of patients or over long periods of time. In order for these studies to move towards standard practice and commercialization, scalable technologies for the isolation, ex vivo manipulation and delivery of these cells to patients must be developed. Additionally, regulatory strategies and clinical protocols for the collection, creation and delivery of cell products must be generated. In this article we will review recent progress in hematopoietic cell and gene therapy, describe some of the current issues facing the field and discuss clinical, technical and regulatory approaches used to navigate the road to product development.

Keywords

Cell therapy; gene therapy; adoptive immunotherapy; stem cells; clinical trials

Introduction

The transition from the laboratory to the clinic (bench to bedside) is well charted for small molecules but less so for cellular therapeutics. Moving a cell product from the basic research laboratory, through process development and onto manufacturing and clinical trials is known as translational research (2) and has become the focus of both federal and private investment. Passage through this proverbial “valley of death” is typically where most candidate therapeutics are stalled, many to never see the clinic. The funding of over 49 Clinical and Translational Science Award centers across the country reflects the NIH view that translational sciences are a high priority in the NIH roadmap for medical research (3). The stages of developing (translating) new therapeutics have been broken down into distinct phases (often termed T1 - T3 activities) to describe the translation from basic science to clinical trials (T1), clinical trials to clinical practice (T2) and broad dissemination to the population (T3)(4). We often only think of the T1 component of this process but all of the

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Author Disclosure Statement:
DD is a Scientific Advisory Board member for Genesis BioPharma and an External Advisor to Owl Biomedical Inc. HPK has no conflicts of interest to disclose

steps are necessary to justify the investment in research made by the Federal Government and private industry.

Like any new medical treatment, the initial years of clinical investigation defined both the utility and limitations of cellular therapy but also led to significant innovation and development of infrastructure in support of subsequent, more advanced studies. For example, bone marrow transplantation was one of the first and still most widely used forms of cell therapy and has helped define both the therapeutic potential of and significant hurdles in developing stem cell products. An important (enabling) developments in cellular therapy was the discovery of a subpopulation of white blood cells expressing the CD34 antigen that contains virtually all of the long-term hematopoietic reconstituting (stem cell) activity in a bone marrow graft (5). The correlation between the number of CD34+ cells transplanted and successful engraftment has helped establish the first stem cell therapy dosing specification to be used in standard clinical practice; a minimum of 2×10^6 CD34+ cells/kg for complete hematopoietic recovery (6). Moreover, CD34+ cells have become the substrate of choice for genetic modification to treat a number of disease indications with an autologous product (7). In a similar fashion, allogeneic transplantation of bone marrow has led to an understanding of the benefits transfer of T-cells with anti-tumor as well as the potentially devastating consequences of T-cell mediated graft versus host disease (GVHD)(8). These latter observations have played a major role in the development of adoptive immunotherapy (AI) strategies for cancer and infectious disease and will be used as examples of how subsequent cell therapies may be developed.

Proof of Concept - Adoptive Immunotherapy

A prominent example of the power of adoptive immunotherapy is the provision of anti-viral immunity following hematopoietic stem cell transplantation where cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenoviral infections are the primary cause of morbidity and mortality (9-11). Since the demonstration of transfer of anti-viral immunity with isolated clones of T-cells (12-14), numerous approaches have emerged to enrich, isolate or otherwise engineer immunity to viruses (14-22). An example is the use of EBV-transformed lymphoid cells lines (LCL) as antigen presenting cells which can be infected with adenoviral vectors expressing both adenoviral and CMV peptides. The LCL then act as antigen-specific feeders during T-cell expansion and result in a population of T-cells with enriched specificity for all three (EBV, Adeno, CMV) viral antigens (23, 24). These approaches have met with reasonable clinical success in controlling CMV and adenoviral infections as well as EBV-associated lymphoproliferative disease (18, 25, 26), although for CMV immunity, the number and identity of CMV epitopes required to confer broad protective immunity is still of significant debate (24, 27). A recent safety report on over 180 recipients receiving over 380 infusions of a range of antigen-specific and/or engineered T-cells indicate that the treatments are safe, without evidence of severe adverse events related to infusion and that close monitoring can be limited to a short period following infusion (28). These studies demonstrate the safety and efficacy of adoptive immunotherapy for a variety of viral pathogens and have resulted in the development of methodologies to prepare and release T-cells for clinical use that have driven the field forward towards GMP compliant production platforms (24, 29). Additionally, regulatory policy and practice at the FDA has been shaped (in part) by the progression of these trials from the laboratory to the clinic and back in an iterative process that helps fine tune the translational infrastructure.

Another compelling and well-tested application of adoptive immunotherapy is the use of tumor-infiltrating T-lymphocytes (TIL) to treat melanoma (30-33). A recent report by Rosenberg et al summarizes the results from three separate clinical trials in which ninety-three patients with recurrent, refractory stage IV melanoma were treated with ex-vivo expanded TIL (34). Patients were infused with the TIL following a lymphodepleting

preparative regimen (cyclophosphamide and fludarabine) ± total body irradiation (0, 2, 12 Gy TBI). (Note: when TBI was administered, patients also received an autologous stem cell transplant). All patients received high-dose IL-2 (720,000 IU/kg) following infusion of TIL to support their proliferation and in vivo activity. Overall (RECIST) response rates were as high as 72% with (durable) complete responses in up to 40% of patients who received the CyFlu + 12 Gy TBI. Interestingly, the highest rate of survival at 5 years was among those patients who had undergone prior immunotherapy with anti-CTLA-4 antibody (Ipilumimab). Ipilumimab blocks CTLA-4-mediated down regulation of T-cell activity and presumably allows for a more sustained anti-tumor response by the infused cells. This series of trials confirms the utility of TIL in treating metastatic tumors but also supports previous evidence that multiple factors work to limit the activity of tumor-associated T-cells in vivo (35, 36). Ex-vivo expansion of tumor-infiltrating T-cells can overcome some of the in vivo anergy induced by the tumor microenvironment but the conditioning of the patient with CyFlu and irradiation creates an in vivo environment favoring homeostatic proliferation and (un-suppressed) expansion of adoptively transferred T-cells and results in more durable complete responses. While this is currently the most promising therapy for melanoma, concerns still exist about the quality of T-cells from patients with large tumor burdens or ‘high antigen loads”(37). Recent evidence demonstrates that T-cells isolated from tumor-infiltrating lymph nodes express higher levels of markers associated with cellular “exhaustion” (apoptosis genes, CTLA-4) which may partly explain the limited ability to generate TIL from some patients and efficacy in less than half the patients treated (38). Nonetheless, similar attempts to isolate TIL from other solid tumors are currently under investigation (39-44).

In a similar fashion, T-cells from peripheral blood of allogeneic donors have been expanded ex-vivo in an attempt to generate allogeneic anti-tumor T-cells for a number of hematological malignancies (45). These studies have demonstrated the therapeutic potential of the approach but graft versus host disease was observed in some patients and remains a significant concern. In a more recent study, investigators looked at the role of vaccination as a way to boost post transplant immunity to tumor through the use of primed autologous T-cells at the time of ASCT (46). Multiple myeloma patients undergoing ASCT were given ex-vivo expanded autologous T-cells collected following immunization with tumor peptides (hTERT, surviving) and pneumococcal peptides (HLA-A2+ patients) while others (HLA-$A2^-$ patients) received T-cells following pneumococcal vaccine only or no vaccine. Accelerated cellular and humoral immunological recovery was observed in patients receiving peptide primed T-cells and vaccination again after transplant (relative to control patients who received unprimed ex-vivo expanded T-cells) with evidence of enhanced immune reactivity to pneumococcal peptides in all patients and additionally to hTERT and survivin in 36% of the multi-peptide immunized patients. However, hTERT and survivin response was not correlated with increased event free survival nor was overall survival different between these groups. Thus, in vivo priming of T-cells followed by ex-vivo expansion and transplant provides enhanced protective immunity to viral infections, but does not always result in improved outcomes with respect to tumor progression. The apparent difference in anti-viral and anti-tumor responses remains a significant hurdle to progress in cancer immunotherapy. In this study, event free survival was correlated with a low level of $CD4^+/FoxP3^+$ regulatory T-cells in the infusion product and in vivo following transfer. This is consistent with the idea that conditions favoring un-suppressed T-cell activity are correlated with improved clinical outcome.

Where anti-tumor immunity cannot be isolated from the existing T-cell repertoire, investigators have engineer specificity into T-cells by transfer of an antigen specific receptor. Primary human T-cells have been successfully modified to express cloned α/β T-cell receptor genes with known specificity and MHC-restriction (47-50) or chimeric antigen

receptors (CAR) that confer antigen specificity in the absence of MHC restriction (51, 52). Antigen specific T-cell receptors can transfer the specificity of a T-cell clone to tumor antigen but the recognition of antigen is restricted the HLA-type of the donor. CAR, on the other hand, are comprised of single chain antibody domains coupled to T-cell signaling domains and recognize native antigen. While some success has been described in the clinical translation of these and other adoptive immunotherapy approaches (53-57) immune responses to transgene-encoded proteins and cellular senescence can limit the persistence of cells following adoptive transfer (37, 58, 59). Improved intracellular signaling in CAR constructs that include a variety of T-cell co-stimulatory signal domains (CD28, 4-1BB or OX40) helps prevent apoptosis and results in more robust proliferation. Several investigations have concluded that provision of a co-stimulatory signal prolongs the survival of T-cells through the increased expression of anti-apoptotic genes and results in enhanced in vivo persistence and anti-tumor efficacy (60-64).

One of the more extensively studied CARs is that for CD19 in B-cell neoplasia (62, 65-70). The potential of two different co-stimulatory domains in CAR modified T-cells for eradicating tumor was recently reported by two independent groups and has established the clinical utility of the approach (71, 72). Bretjens et al evaluated the use of autologous CD19-CAR28ζ T-cells (expressing both CD28 and CD3ζ signaling domains) to treat CD19+ chronic lymphocytic or acute lymphoblastic Leukemia (CLL or ALL) patients. The CD19-CAR28ζ T-cells homed to bulky tumor in vivo, persisted and retain cytolytic activity for 8 days. Importantly, this effect was only observed in patients with prior lymphodepleting chemotherapy. Three of five evaluable patients (those who received lymphodepleting chemotherapy) showed reduced or stable disease lasting from 2-6 months. In a similar study, a patient with CLL who's disease was progressing following Rituximab was treatment received 3×10^8 CD19-CAR(ζ/4-1BB) modified T-cells following lymphodepleting chemotherapy. While the patient experienced immune response-related events within the first week following infusion (fever, chills, rigors etc.) and tumor lysis syndrome at 22 days, by day 28 the tumor adenopathy was no longer palpable and there was no evidence of CLL in the bone marrow. Sustained remission was reported at up to six months following treatment. The highest level of CD19CAR+ T-cell in the patient was at the time of tumor lysis syndrome and represented over a three log expansion of the infused cells. Importantly, while the CD19CAR+ T-cells were detected for up to six months after infusion, the total number of cells contracted over ten fold, demonstrating a controlled immune response to antigen. These studies demonstrate that the provision of a co-stimulatory signal leads to sustained objective responses in patients treated with CAR-modified T-cell products and reinforces the requirement for prior conditioning of the patient.

Taken together, the recently reported results from adoptive immunotherapy trials have demonstrated the potent anti-viral and anti-tumor response of T-cells following in vivo priming, ex vivo genetic modification and expansion. It is now clear that lymphodepleting preparative regimens are important to success and that the persistence of the cells can be mediated by sufficient signaling to prevent apoptosis (in vivo IL-2 or co-stimulatory molecules). While the endogenous repertoire of T-cells found in tumor sites has been shown to be sufficient for isolating anti-tumor T-cells, there is little control over the composition of the product and the reasons for failure to provide clinical benefit in most patients are still unknown. Thus, the potential of gene-modified T-cells into which the specificity can be “engineered” is extremely important and has yet to be fully tested. The development of an engineered T-cell is entirely dependent on identifying an optimal T-cell substrate for genetic modification and to ensure sufficient persistence to provide therapeutic activity in vivo. Several groups have now identified naïve (73-75), central memory (60, 69, 73, 76, 77) and central memory stem cells (75, 78) as interesting cell substrate candidates for more in depth investigation in this area. Clinical trials are now underway to test the clinical utility of gene

modified central memory T-cell populations (S Forman personal communication) and more are in the planning stages.

Proof of Concept - Stem Cell Gene Therapy

The last ten years have also seen a dramatic increase in the number (and success) of stem cell based gene therapy trials. Allogeneic stem cell transplantation has been used in proof of principle studies in patients with inborn immunodeficiencies (79, 80), or metabolic disease (81) demonstrating disease correction via a cellular carrier of a wild type copy of the relevant missing or mutated gene. More recently, allogeneic bone marrow transplantation of an HIV positive patient with HIV-resistant (Δ32 CCR5$^{-/-}$) donor stem cells resulted in long term cure of HIV, demonstrating the genetic transfer of infectious disease resistance through a stem cell product (82). These studies have provided proof of concept support for gene replacement to correct monogenic or immunological disease but have also outlined important limitations of the allogeneic transplant approach. The combined morbidity and mortality of allogeneic transplantation (specifically graft versus host disease) and the low frequency of matched related or fully allogeneic donors available for such a procedure precludes the widespread application of this approach. It follows that cellular engineering of autologous products to provide metabolic function, disease resistance or anti-tumor activity would be a more feasible approach for most patients.

With the knowledge and technology developed around bone marrow transplantation, numerous groups initiated clinical investigations using autologous gene-modified HSPC to correct monogenic disease. Clinical gene therapy studies have been performed in children with X-Linked or ADA-deficient severe combined immunodeficiency (SCID), Wiscott-Aldrich Syndrome, β-thalassemia and Fanconi anemia (see below). Early clinical studies in ADA-SCID were conducted using gamma-retroviral vectors expressing the human ADA gene to genetically modify autologous bone marrow or cord blood-derived HSPC (83-85). The outcomes have been promising, resulting in restored immunity and independence from PEG-ADA enzyme replacement therapy with long term follow-up and independent validation of the approach recently reported (86, 87). Attempts to replace the genetic deficiency in X-Linked SCID (IL-2 receptor common gamma chain) via retroviral gene therapy also showed clinical benefit, but resulted in a high rate of leukemia as the result of insertional mutagenesis by the vector (88, 89). In a more recent report by Gaspar et al (90), 10 pediatric patients undergoing HSPC gene therapy for X-linked SCID demonstrated variable but sustained immunological function with only 1 patient developing a T-cell acute lymphoblastic leukemia as a result of the process. Comparable clinical efficacy has not been observed in adults undergoing similar therapy (91). Similar results where clinical efficacy was offset by troubling transgene insertion patterns and clonogenic dominance have been observed in patients transplanted with retroviral vector-modified HSPC for Wiskott-Aldrich syndrome (92) and chronic granulomatous disease (93, 94).

Thus, despite promising clinical results, gamma retroviral vectors are no longer considered for most indications (ADA-SCID notwithstanding) due to the high risk of transformation or progression to myelodysplasia in these patients.

Following this initial series of trials and in consideration of the transformational activity of the gammaretroviral vectors, a series of gene therapy trials have demonstrated safe efficacious genetic modification of HSPC using lentiviral vectors. The lentiviral vectors used in these trial are derived from HIV but have had many essential genes removed (gag, Pol, Env) to prevent viral replication in vivo. The viral coat glycoprotein has been replaced with the vesicular stomatitis virus G-protein (VSV-G) to broaden host range (including cells of the hematopoietic lineage) and the viral long terminal repeat regions have been engineered to be self-inactivating, that is they are deleted upon viral integration in the

genome, rendering the virus replication incompetent. Two pediatric patients with X-Linked adrenoleukodystrophy were treated with HSPC that had been transduced with a lentiviral vector encoding the ABCD1 transporter protein (the protein that is defective in adrenoleukodystrophy) (95). The treatments resulted in objective clinical responses an arrest of progressive cerebral demylenation and stabilized or improved neurological function following treatment. Subsequent analysis of lentiviral insertion sites revealed that the transgenic viral integration patterns were localized to specific genomic regions but were not transforming (96).

A similar study in β-thalassemia has demonstrated transfusion independence in a single adult subject 3 years following transplantation with HSPC modified by a lentiviral vector expressing adult β-globin under the control of the endogenous β-locus control region cloned into the vector (97). Interestingly, the integration of the transgene within the third intron of the high mobility group AT-hook 2 (HMGA2) gene led to increased expression of the transgenic β-globin sequences and accounted for most of the therapeutic β-globin expression even though cells with this integration pattern represented 2-8% of the blood cells at 28 months. It was noted that HMGA2 expression was elevated >10,000 fold in cells with HMGA2 intron 3 integrated vector. So, despite the general safety of the procedure, significant concern about integration sites of lentiviral vectors remain. Nonetheless, successful gene therapy trials in adults treated for hemophilia B (98) and Leber congenital amaurosis (99) have also been reported and the number of trials demonstrating general proof of principle continues to expand.

However, applications of gene therapy have proven to be more complicated when providing systemic treatments for non-life threatening diseases. Our own studies in HIV gene therapy at City of Hope were conducted in HIV patients undergoing autologous stem cell transplantation for progressive lymphoma (100, 101). The purpose of the study was to determine the safety and efficacy of RNA-based HIV inhibitors following transplant of gene-modified CD34+ hematopoietic stem and progenitor cells (HSPC). Our results demonstrate gene marking and anti-HIV gene expression (siRNA and ribozyme) in the peripheral blood and bone marrow of treated patients for up to 36 months without evidence of clonal dominance or leukemia. The frequency of gene modified cells was low in these patients due to the ethical requirement for infusion of an (unmanipulated) backup HSPC product until the gene modified HSPC were demonstrated to result in robust hematopoietic engraftment. Subsequent studies are underway to evaluate the level of gene marking when only the gene-modified cells are infused. Interestingly, a transient increase in gene marking was seen following transient viremia in 2 of our patients and mirrors the putative selection of gene-modified cells by virus seen in both animal models of HIV gene therapy and prior clinical investigation (102, 103). One patient is scheduled for a temporary structured treatment interruption of his HAART therapy during which viral load and the levels of gene-marked cells will be followed. In order for this approach to be transferred to the non-malignant HIV population (the ultimate target market) non-ablative conditioning regimens will have to be developed and bone marrow may have to be considered as a source of CD34+ cells as the cost and risks of harvesting mobilized peripheral blood may outweigh the benefit of the procedure.

The Path Forward

With the described clinical trials having demonstrated proof of concept for cell and gene therapy, we are now facing the bigger task of moving candidate therapeutics into larger phase II trials to ask about the overall benefit in a more formal, controlled fashion. During early phase clinical trials, it is common for investigators to manufacture cell products using (qualified) research grade tissue culture devices and reagents as long as they are sterile,

endotoxin free and do not pose a significant risk to the patient receiving the product. Additionally, many processes are conducted with open steps such as feeding of cells in culture, repeated centrifugations, transfer between vessels etc. Product characterization may be limited to the expression of one or two cell surface markers (i.e. CD3, CD8, CD11b, CD34) and in many cases, potency assays are limited to viability and cell count but do not address the biological activity of the product as it relates to anticipated mode of therapeutic action (cytokine secretion, cytotoxicity, proliferative potential, transgene expression etc.). As products move from pilot or phase I trials to larger phase II/III trials, investigators must do more to meet current federal good manufacturing practice (cGMP) requirements include auditing and qualifying reagent manufacturers, developing closed cell processing procedures, developing and qualifying potency assays, performing product stability and distribution (shipping) studies and extensive safety testing for master and working cell banks and viral seed stocks. Additionally, laboratory information management systems (LIMS) will be required to track data on raw material, products and patients and are currently being developed using available tools (104). The FDA provides guidance documents that describe expectations for biologics manufacturing as products move through the clinical trial process. Links to important government guidance documents are provided in Table 1. Additionally, extensive information is available for those interested in resources for and advancements in cell therapy through the ISCT website (www.celltherapysociety.org) and a user-based blog known as the Cell Therapy Blog (http://celltherapyblog.blogspot.com/).

Typical phase II trials involve larger patient cohorts (up to several hundred) and are designed to establish dosing and efficacy of the candidate therapeutic as well as extend data on safety. When cells (or gene-modified versions) are the therapeutic candidates, the biggest problem may be scaling “out” the process from one that easily produces cells for 5-10 patients to one where many more (50-100), simultaneous, patient-specific products may be manufactured at one time. Few academic centers or small biotechnology companies have the capability to manufacture cell products but numerous contract manufacturing organizations are available to support those without the internal infrastructure (Table 2). For academic investigators, the NHLBI supports the Production Assistance for Cellular Therapies (PACT) program which is an excellent manufacturing resource for most academic centers working from federally funded grants. PACT also provides regulatory guidance and statistical data collection on products. More information on the process can be found at the PACT website (www.pactgroup.net). Other NHLBI resources include vector manufacturing and toxicology testing through the Gene Therapy Resource Program – GTRP (www.gtrp.org) and the Science Moving towArds Research Translation and Therapy – SMARTT program (www.nhlbi.nih.gov/new/SMARTT.htm) which identifies translational resources for investigators seeking assistance.

Cell Processing and Culture

Perhaps the most important step in cell therapy is isolation of the cell substrate. Cells may be isolated from a patient (autologous) or a healthy volunteer (allogeneic) or be derived from a single source and divided into multiple individual doses (master cell bank). Primary cells harvested from patients (tumor, blood, marrow) may need to be washed free of undesired cells (fat, platelets red blood cells) and enriched for a target cell population (T-cells, dendritic cells, stem cells). Many procedures will also require multiple steps involving genetic modification and expansion (see below). Procedures employed during pilot and phase I clinical investigations are typically performed manually, using methods, reagents and devices that are not amendable to scale-up or do not fully meet clinical quality requirements. Examples include the use of T-flasks to grow cells, ruminant animal derived materials such as serum, trypsin and gelatin, and centrifugation to wash, concentrate and

formulate samples. To address many of these issues, new technologies are emerging that support process scale-up, automation and reduction of technical time and error.

Several groups have described automated closed systems for cell washing and formulation that have been used successfully for clinical cell processing including closed system density separations and elutriation (105-110). These large batch and continuous flow technologies are automated, include sterile disposable plastic tubing sets, and support scaling to clinical levels of cells (>10e10). Where these technologies have been adapted from blood banking or other regulated industries, the devices often meet regulatory requirements for instrumentation used in the manufacturing of clinical materials. There is however opportunity to develop newer devices to address tissue specific processing issues such as harvesting cells grown on matrixes or artificial scaffolds.

Selective enrichment and manipulation of cells with magnetic beads has been an enabling technology for the development of clinical cell therapy applications. There are two clinical scale magnetic cell selection systems currently used in clinical cell therapy applications; the CliniMACS® system (Miltenyi Biotec, Bergisch Gladbach, Germany) and Dynabeads® CD3/CD28 CTS™ (Life Technologies, Carlsbad California). The CliniMACS® device employs small paramagnetic particles coupled to antibodies to selectively enrich cells based on cell surface antigen expression or IFNγ secretion in response to specific antigens. Current clinical applications include (but are not limited to) the isolation of CD34+ cells for allogeneic transplantation (111, 112) and gene therapy (86, 95, 100), T-cells for adoptive immunotherapy (27, 113, 114) and monocyte/dendritic cells for vaccine trials (115). The larger magnetic beads (Dynabeads®) have also been used extensively in cell selection and expansion. The co-localization of antibodies to the T-cell receptor CD3ε domain and an antibody to the co-stimulatory CD28 molecule on the bead presents a powerful proliferative signal to T-cells and has a long standing record of use in clinical T-cell expansion studies (45, 46, 116-120). Both companies provide limited clinical reagent sets but beads ready for antibody conjugation are also available for customized application. Both companies offer access to their systems to qualified investigators via a letter of cross reference to a master file on record with the FDA. An advantage of bead selection is the rapid processing of a large number of cells in a short amount of time. However, continual refinement of the definition of a hematopoietic stem cells (121), the identification of and debate over what T-cell population is the best substrate for durable engraftment and antitumor activity (74, 77, 122, 123) have driven the field towards multiparameter isolation of cells for therapeutic applications. Additionally, cells expressing low densities of antigen will bind many fewer beads and are thus difficult to isolate by magnetic separation. All of these issue can (and have been) addressed using multiparameter, fluorescence-activated cell sorting.

Currently, there are a limited number of fluorescence-activated cell sorters being used for clinical applications and most are adaptations of research machines. The Influx® cell sorter (BD Biosciences, San Jose, CA) will isolate cells at 25,000-75,000 cells per second based on up to 16 parameters. It is equipped with a disposable fluidics kit to allow for easy changeover between patient samples and a HEPA filtered cabinet to contain aerosol generation during sorting. Its small footprint allows for installation in minimal spaces compared to conventional cell sorters. However, sorters like the Influx are expensive, required highly trained operators and are significantly complex with realistic potential for “process deviation” during long sort runs. Additionally, since they generate aerosols and cells are sorted into open tubes or plates, the ultimate clinical application of this technology awaits smaller, closed-system sorting devices. Newer generation cell sorters based on microelectromechanical (MEMS) technology are currently in development (www.owlbiomedical.com) and address some of these aforementioned limitations of the research devices.

Cell culture also suffers from the limitation of technologies designed for research with limited potential to scale up for clinical production. As mentioned above, multi-well plates and T-flasks are often used to propagate cells in early stage clinical trials but these devices are not scalable, are subject to the introduction of contaminants during manual manipulations and are static in nature, requiring batch feeding and resulting in limited cell densities. In order to address these and other (regulatory) issues and move towards larger scale clinical studies, numerous labs have successfully transferred cell culture processes from flasks and plates to gas permeable culture bags or closed system bioreactors (29, 55, 124-126). Culture bags can be customized to include any number or configuration of sterile weld, luer or quick connect ports to facilitate sterile closed transfer of products between vessels. Culture bags from 5 mL to 100 liters are manufactured by several companies (American Fluoroseal Corporation, Charter Medical and Origen Biomedical) according to the requisite standards for use in cell product manufacturing. Additionally, sterile tubing sets, media bags and centrifuge tubes can be used to connect culture bags and other processing devices using tubing welders and sealer designed for the blood banking industry. These techniques facilitate addition or removal of media, growth factors or cells in a closed system and thus reduce the potential for adulteration of products during handling. With the ability to have all media, components, buffers and solutions manipulated in closed systems the incidence of contamination of cell therapy products has been significantly reduced. Thus, closed system cell culture bags have had a significant impact on meeting regulatory requirements related to product safety. Newer disposable devices such as the G-Rex flask (Wilson-Wolf Manufacturing, St. Paul, MN), provide enhanced gas exchange over standard tissue culture flasks have also been shown to dramatically improve the densities of cells in static culture. While not yet a completely closed system, this technology may find a specific niche such as growing solid tissue fragments (to generate TIL) or other 3 dimension culture applications (127).

At a larger scale, perfusion bioreactors like the WAVE (GE Healthcare, Fairfield, CT) have been widely applied for cell therapy. The Wave is a disposable culture bag system has been used mostly for growing suspension cells and contains programmable controllers for media and nutrient perfusion. Using this system, cell densities 10 fold higher than static cultures can be achieved (70, 126, 128, 129). Alternatively, a hollow fiber bioreactor that can support the growth of either suspension or adherent cells has been developed (Quantum® Cell Expansion System, Caridian BCT, Lakewood, CO) and is currently undergoing clinical evaluation for mesenchymal stem cell production. The automated system includes a closed culture disposable set for media addition, gas exchange and harvesting product. The provision of these types of bioreactors allow for scaling cell culture to clinical levels, controlling the process in an automated fashion and complying easily with regulations related to ensuring the safety of products.

For some cells (especially HSPC), ex-vivo expansion is not a straight forward process. During extended cell culture, HSPC can differentiate into cells with reduced ability to engraft the bone marrow. Over the past few years, numerous strategies have emerged to expand stem cells using a wide variety of approaches including Notch Delta interactions (130, 131), homeobox genes (132, 133) and an aryl hydrocarbon receptor antagonist (134). Most expansion strategies use expansion of CD34+ cells as a metric but some also employ engraftment of immunodeficient mice as a measure of the stem cell content of an ex-vivo expanded product. What has become clear is that even the immunodeficient mouse models of hematopoiesis may not predict clinical outcome due to the complexity of homing and engraftment of human cells. Comparisons of engraftment between immunodeficient mice and non-human primates have shown disparities in the long term repopulation of the animals with stem cells as determined by common gene integration sites in cells of the lymphoid and

myeloid lineages (135, 136). Development of these strategies will require additional investigations before ready for the clinic.

Genetic Modification

Among the lessons learned in the studies described above, perhaps the most prominent has been that stable integration and expression of vector-encoded transgenes into primary cells is a rare event and can lead to disrupted gene expression and leukemia. While pseudotyped lentiviral vectors have demonstrated excellent efficacy in transducing a large number of primary cell types and possess a better safety record (to date) than retroviral vectors (137), they are not without limitations (138). Lentiviral vectors are typically produced in a transient fashion by transfection of a human embryonic kidney cell line (HEK 293) with 3-4 helper plasmids encoding essential viral elements (gag, pol and env) and the plasmid carrying the vector backbone and therapeutic insert (139). The productions are transient because the proteins required to produce and package virus are toxic to the producer cells (p24, VSV-G) (138). This means that a new batch of plasmid and 293 cells must be used for every lot of vector manufactured. Variability in the relative transduction of each plasmid can result in significant differences in viral titer and quality and are not amenable to large-scale, repeated clinical production. Once produced, the vectors must be concentrated and formulated for both cryopreservation and transduction of target cells. Ongoing efforts to improve productivity of lentiviral vectors for clinical use include the creation of stable packaging cell lines (140-143), electroporation of suspension cultures of 293 cells (144), regulated expression of the toxic accessory proteins (145-147) or combinations of the above(146). Other strategies to improve vector quality have been aimed at concentration and purification of vectors by ultrafiltration and chromatography (148). We continue to be hopeful that these advances viral vector production will be incorporated into the manufacturing of clinical lots over the next 5 years. It is also very important that these tools are made widely available and not restricted by cost or proprietary considerations.

Genetic modification of cells has also advanced since the early gene therapy studies. In addition to gene replacement, methods to silence gene expression by deletion of genomic sequences or repair mutant genes by directed homologous recombination have been reported (149-154). Zinc fingers are DNA binding proteins that can be engineered to bind to DNA in a sequence specific fashion. Fusion of Zinc Fingers with the catalytic domain of a type II restriction enzyme (Fok1) creates a zinc finger nuclease that is capable of making sequence specific cuts in the genome of a target cells (155). The DNA repair mechanism of the target cell attempts to repair the genomic damage using an error-prone mechanism (non-homologous end-joining) which leads to deletions that result in elimination of the open reading frame and thus expression of mature protein. Repair can also result in corrected point mutations when a wild type donor template is included (149). The technology has also shown excellent potential in for clinical implementation by targeting the elimination of CCR5 gene expression for HIV gene therapy (156, 157), correcting point mutations in sickle cell anemia (158), hemophilia (159) and α-1 anti-trypsin deficiency (160). Despite the demonstrable efficacy and progress in the clinic, ZFN strategies have come under great scrutiny regarding toxicity and the potential for off-target cutting (161-164).

Two similar but distinct genomic editing (meganuclease) technologies have been described and are meant to address some of the limitations of the ZFN technologies. Transcription activator-like effector nucleases (TALENS) have been reported to be as catalytically active as some but not all ZFN and have other limitations (size and ease of delivery) that currently limit their use in clinical applications (151, 165, 166). Another emerging family of gene modifying nucleases are the homing endonucleases derived from a large family of short intronic elements or “inteins” that can be engineered to mediate highly specific genomic cutting in a wide variety of target genes (167). These relatively small proteins can be made

highly specific and have been shown to disrupt or repair a variety of gene sequences including RAG-1 and dystrophin (168) and may fill biotechnological niches that are not effectively addressed by ZFN or TALENS.

Selective degradation of mRNA using small interfering RNA (siRNA) or micro-RNA sequences has also been reported as a way to control endogenous gene expression (169-174). RNA expression has the advantage of being non-immunogenic and thus is an advantage over protein-based inhibitors. The expression of siRNA inside of target cells can lead to the destabilization of disease promoting gene expression (175), elimination of viral receptors (176, 177) or induction of cell death by targeting essential cell survival genes in regulated manner(175, 178). Placement of micro-RNA sequences in the 3′ region of an expressed transgene can limit expression in antigen presenting cells and thus avoid immune recognition of therapeutic (transgenic) proteins (179). These new tools have the potential to eliminate the need for randomly (or dangerously) integrating viral vectors.

It is often the case that viral vectors are deemed too risky, too expensive, too toxic or are not effective for the particular application of molecular delivery. Several reports (and clinical trials) include the use of suicide genes or inducible apoptosis systems to act as safeguards when, despite the best safety efforts) cells go awry and threaten to result in malignant disease or other life threatening clinical complications (180-182). Otherwise, closed non-viral systems for introduction of DNA and peptides to dendritic cells and NK T-cell by electroporation have been described (183, 184) and obviate the need for viral vectors in some applications. Other systems such as nanoparticles and lipid-based complexes have also been described and are currently being developed for clinical implementation (185-188). Another promising technology being developed for genetic modification of cells is the use of transposable genetic elements or Transposons. Transposons are ancient genetic elements that utilize homologous recombination as a method for introducing genes into target cells at homology-defined sites. One noteworthy example is the use of the Sleeping Beauty transposon system to modify CD34+ blood stem cells and T-cells, the latter of which is rapidly progressing through pre-clinical development and into clinical trials(185).

One of the major advantages of these novel genetic editing technologies is the potential to avoid integration-mediated oncogenesis by prospectively inserting transgenic sequences into “safe harbors” where transforming sequences are not present (189-192). A limitation of this approach is the low efficiency with which modifications are made, generally requiring a selection step or selective growth advantage of the modified cells to enrich for cells with therapeutic potential. Significant advances in efficiency are thus required before these techniques are applicable to most gene therapy applications.

The Next Chapter

As cell therapy product move from proof of concept towards more advanced (Phase III) clinical trials, the need for specialized production, clinical and regulatory professionals increases dramatically. These include process development scientists, quality systems and regulatory affairs coordinators, protocol nurses, blood bank or surgical teams, primary care (referring) physicians, clinical research associates, program managers, product development staff and others. These individuals will need specialized training in the use of cells as therapeutic products in order to support successful implementation. For example, at City of Hope we have developed a job description for a Cell Pharmacist in recognition of the fact that administration of cell products has all of the requirements of pharmaceuticals (patient eligibility, dose verification, review of contraindications etc.) as well as specialized requirements like final product formulation and filling of specialized delivery devices. The California Institute for Regenerative Medicine (CIRM) has supported extensive

development of cell therapy infrastructure in California and proffered the idea of an alpha cell therapy clinic to provide centralized facilities with the requisite infrastructure to support the translation of promising new cell based therapies to the clinic (193, 194). Together, these resources and progressive planning are having a significant impact on current cell and gene therapy trials.

As cell products move even further towards commercialization, a clean separation of the clinical research aspects of cell therapy will give way to product development concerns and regulatory compliance. It is expected that process changes will occur in order to control cost of goods, ensure product quality and develop product marketing and distribution systems. Validation of manufacturing procedures and product qualification assays may require significant technology development. Also, the ability to receive a cell product from a patient, ship to and process at a central facility and return the product to the patient in a timely, qualified and efficacious fashion will determine the feasibility of widespread application of any personalized cell product. The regulatory requirements in this area are just beginning to take shape as the first cell therapy products are brought to market.

Conclusion

Recent advances in cell and gene therapy have demonstrated the proof of concept that cells are potent agents for treating monogenic, infectious and neoplastic disease. Moreover, cell therapy potentially may provide a single dose, long term solution to disease intervention that may rapidly outpace the current more transient treatments such as chemotherapy, radiation therapy, protein replacement (ADA) or long term anti-virals (HAART) as the primary treatment for disease. The recent development of infrastructure and advances in the understanding of the physical and biochemical processes that govern cell isolation, expansion and genetic modification promise to launch cell and gene therapy onto the forefront of medical care. Significant investment in cell therapy by State and Federal government agencies and the biopharmaceutical industry will be required to implement the widespread dissemination of what are now considered “boutique” therapies if we are to advance from proof of concept to acceptance in the medical community. Only when physicians have a Current Procedural Terminology (CPT) code for reimbursement of cell therapy procedures offered can we claim “mission accomplished”.

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Table 1

Links to important Cell and Gene Therapy Guidance Documents

Cell and Gene Therapy Guidance:	**http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/defau**
Process Validation Guidance:	www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070336.pdf

Table 2

Contract manufacturing organizations for biologics production.

Organization	Web URL
Lonza	www.lonza.com
PX'Therapeutics	www.px-therapeutics.com/px_cGMP_manufacturing.php
Omnia Biologics	www.omniabiologics.com/
Therapure Biopharma	www.therapurebio.com/
Progenitor Cell Therapy	www.progenitorcelltherapy.com
SAFC	www.safcglobal.com
Florida Biologix	www.floridabiologix.ufl.com
Waisman Biomanufacturing	www.gmpbio.org