Chapter 6
Production of Clinical T Cell Therapies

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Abstract The improving effectiveness of adoptive T cell therapies has led to their increased clinical application. Most of these adoptive T cell therapies are being produced in small lots in cell therapy centers affiliated with or located within academic health centers. Typically, the cells are produced from autologous or HLA compatible donors and one lot is used for a single patient. As part of early phase clinical trials, the best available methods and devices for the manufacture of clinical grade T cell therapies are described. For most adoptive T cell therapies the starting material is a peripheral blood mononuclear cell (PBMC) product that is collected by apheresis using closed system blood cell separators. Many manufacturing processes require that red blood cells be removed from the PBMCs or that T cells or T cell subsets are isolated. Classically, T cells have been cultured in flasks, but culture in closed systems which reduces the risk of microbial contamination is desirable and bags and bioreactors are often used for T cell culture and expansion. T cell culture involves growth and expansion in media supplemented with serum, cytokines and feeder cells or other artificial stimulators, i.e. anti-CD3/28 beads or K562 cell line. Recently, closed system transduction methods have been developed that can be used to produce genetically engineered T cells. Automated instruments are available to wash and concentrate products. The final product is assessed for the quantity of cells present, purity, sterility and potency. The use of these best practices is allowing for the consistent manufacturing of high quality cellular therapies to support early phase clinical trials.

Keywords Adoptive cell therapies • Good manufacturing practices • Cell culture • Cell selection • Tumor infiltrating leukocytes • Genetically engineered T cells • Cell processing • Chimeric antigen receptor T cells • High affinity receptor T cells

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Introduction

Adoptive cellular therapy using T cells is becoming more effective and its use is growing. For many years tumor infiltrating lymphocytes (TIL) have been isolated from metastatic melanoma lesions, expanded and given as an effective anti-tumor autologous therapy [1]. The discovery that the administration of leukocyte reductive chemotherapy and irradiation therapy prior to TIL infusion enhances their clinical effectiveness is leading to the wide spread use of this therapy [2, 3].

The use of other adoptive T cell therapies for cancer is also growing rapidly. Autologous peripheral blood T cells are being genetically engineered to produce potent anti-cancer cells. T cells can be genetically engineered to express high affinity T cell receptors (TCR) and chimeric antigen receptors (CAR) that are specific for antigens expressed by tumors [4]. The transduction of autologous T cells with high affinity TCRs reactive with melanoma specific antigens allows for the treatment of melanoma patients when they do not have metastatic lesions that can resected for TIL production or when TIL cannot be cultured from a resected lesion. In addition, autologous lymphocytes engineered to express high affinity TCRs are being used to treat types of metastatic cancer which have not been treated with TIL. T cells expressing TCRs specific for the fetal embryonic antigen NY-ESO-1 are being used to treat patients with metastatic melanoma and metastatic synovial cell sarcoma [5] and T cells with TCRs specific for MAGE-A3 are being used to treat patients with metastatic melanoma, synovial cell sarcoma and esophageal cancer [6]. CAR T cells are engineered to express a vector encoding the zeta chain of CD3, the single change variable region of an antibody directed to a tumor antigen and a co-stimulatory molecule. CAR T cells specific for CD19 antigen have proven to be very effective for treating B cell malignancies including: acute lymphocytic leukemia, chronic lymphocytic leukemia and lymphoma. CAR T cells specific for CD20 have been shown to have clinical activity in patients with B cell lymphomas [7] and those specific for GD2 in patients with neuroblastoma [8]. CAR T cells directed to other antigens are also being developed including CD22 [9], CD23 [10], CD70 [11], immunoglobulin kappa light chain [12], B-cell maturation antigen (BCMA) [13] and erythropoietin-producing hepatocellular carcinoma A (EphA2) [14].

An important aspect of producing cell and gene therapies involves the use of good manufacturing practices (GMP). GMP is a system of evolving practices that have been found to yield safe, high quality products. GMPs involves the entire process of producing cell therapies from the screening, testing, and selection of the donor; to cell collection and processing; and to cell administration and recipient follow-up. It is a common misconception that GMP only means manufacturing cells in a highly controlled facility specifically designed for this purpose. However, it also involves maintaining a group of qualified and properly trained staff; developing and adhering to standard operating procedures; appropriate evaluation and testing of the starting materials, reagents, intermediate products and the final product; qualifying the donor and vendors supplying materials and much more. Many aspects of GMP production of cell and gene therapies are well worked out. For example to qualify

that the donor of starting materials there are screening questions that the donor must answer and blood tests that must be performed to minimize the possibility that the donor may have an infectious disease that could be present in the starting material and which could be transmitted to the recipient. These requirements have been standardized and tests are readily available. However, the requirements concerning GMP manufacturing of cellular therapies are less clear because the best methods of manufacturing have yet to be defined. This is due in part to the evolving nature of cellular therapies. New cell therapies are being rapidly developed and existing therapies are constantly being modified.

Most T cell therapies produced at academic centers are manufactured under INDs for phase I and phase II clinical trials. Most of these cell therapies never make it past phase I or II trials since they are either not effective or not safe and as a result, academic centers are constantly developing new therapies. Since many new cell therapies will fail, many laboratories at academic centers do not spend as much resources and time to develop manufacturing processes for products used in a phase I/II clinical trials as for products used for later phase trials. As a result many protocols taken to phase I and II trials use the best available but not necessarily the best possible reagents and methods. Using the best available processes and reagents allows a protocol to be taken from the research laboratory to the clinic in a reasonable amount of time and allows for the timely evaluation of new therapies. Using the best possible methods for phase I and II trials may require a very lengthy duration of time to develop a cell therapy that in the end is not effective. For early phase trials it would be ideal to use the best possible methods, but it is reasonable to use the best available manufacturing processes that will allow the production of cells to begin in a timely manner so subject accrual can begin in order to determine if the product is safe and has any clinical efficacy. However, if the results of early clinical trials are promising, it's important to quickly convert the methods used for manufacturing the phase I/II products to those most compliant with GMPs and those that are appropriate for the higher expectations associated with late phase products. If necessary, efforts should be made to develop new GMP compliant methods, devices and reagents since any products that will go on to phase III trials and commercialization are expected to be manufactured using methods that yield the safest, most consistent and most effective products.

There are problems associated with not using the best possible methods for the production of early phase clinical trials. The re-engineering of early phase cellular therapy manufacturing processing procedures to meet the more demanding requirement of late phase products is generally very time consuming and costly since cell therapies are very complex. Cell therapies make use of difficult to obtain starting materials such as cells and vectors, include many steps and often require prolonged cell culture and expansion. T cell therapy manufacturing protocols generally take 1 or 2 weeks or longer to complete. Due to the complex nature and long duration of the manufacturing protocols it often takes a year or more to re-engineer and validate a cell production processes and multiple cycles of changes may be required to convert a manufacturing process used in phase I/II trails to a more robust, reliable and cost effective manufacturing processing.

This chapter will describe the various steps involved in manufacturing T cell therapies and the current best practices. The chapter is focused on the manufacturing of cells for a single patient; either for autologous use or allogeneic cells collected for administration to a specific patient. These patient specific allogeneic cells are generally from HLA-compatible relatives or unrelated donors. This type of manufacturing is usually preformed at academic health centers and typically involves phase I/II clinical trials. The size of each lot of autologous or allogeneic directed donor product is generally small, only large enough to treat one patient. Methods to manufacture consistently high quality autologous and directed donor T cell therapies that are compliant with GMP and that make use of resources available at cell processing laboratories in academic centers are described. The use of these practices helps academic cellular therapy centers produce consistently high quality products which allows for the better assessment of the efficacy of early phase products and allows for a better transition to phase III trials and licensure.

Supplies and Reagents

A wide variety of media, media supplements, cytokines and growth factors are used to produce cellular and gene therapies. The quality of these reagents has an important impact on the consistency and safety of the final cellular or gene therapy product. For example, many research laboratories use fetal bovine serum (FBS) as a media supplement to support the growth and expansion of cells in culture, but FBS can negatively impact the safety and quality of cellular therapies. There is considerable amount of lot to lot variability in the ability of FBS to support cell proliferation. Even if a laboratory goes through great lengths to select lots of FBS with similar ability to support the cell of interest, considerable differences may occur in the cultured cells when a new lot of FBS is used. The use of FBS also presents a potential safety issues. The use of animal products and animal derived reagents in cell manufacturing exposes the product recipient to risk of acquiring a xenogeneic infection. In addition, it is difficult to entirely remove foreign proteins from cells cultured in FBS and some recipients will develop immune responses including anaphylaxis due to IgE antibodies to bovine proteins if they are repeatedly exposed to cells cultured in FBS [15–17]. As a result, it is best to avoid the use of all animal derived reagents in the manufacturing of cellular therapies.

Media, media supplements, cytokines and growth factors that are xeno-free are available. From among the xeno-free reagents it is important to select the highest quality reagents. Most reagents are available in several different grades ranging from pharmaceutical grade to research grade. The highest quality reagents are of the highest purity and contain the least contaminants. They are also manufactured under the most stringent conditions and tested more rigorously to ensure that they are consistently of a very high quality. Some cytokines and growth factors such as IL-2, interferon-γ, granulocyte colony-stimulating factor and granulocyte macrophage-colony stimulating factor are available as pharmaceutical grade reagents. Many other cytokines and growth factors are available as GMP grade

reagents made specifically for the manufacturing of clinical cellular therapies. Some cellular therapy laboratories use cytokines and growth factors that are less than GMP grade since they are less costly, but this practice is not recommended since it could lead to cellular and gene therapy products of inferior quality.

Closed System Processing

One of the most important concepts of manufacturing cellular therapies is to maintain the cells in a system that is closed to the external environment. Since cell and gene therapies cannot be sterilized at the end of manufacturing, it is important to maintain the sterility of the products throughout the collection and manufacturing process. In order to prevent microbial contamination of cell therapies, as much as possible, closed systems should be used in the collection and manufacturing process. However, most early phase cellular therapies produced at academic centers often move directly from research laboratories to cell processing laboratories and research laboratories almost always use systems that are open to the environment for cell culture: T flasks, multi-well plates, and tubes. Therefore, one of the challenges for clinical cell processing laboratories is to convert open culture systems into closed systems.

Culture bags can be used to create closed systems. These sterile, plastic, one time use bags are manufactured with tubular tails (Fig. 6.1). Sterile tube welders are commercially available which sterilely connect tubing from one bag to tubing from another bag (Fig. 6.2). This technology allows cellular therapy laboratories to construct customized networks of bags for cell expansion, separation, washing and concentration. It has been used widely in blood centers and blood banks for collecting and processing whole blood, red blood cells, platelets and plasma. To separate bags the tubing connecting the bags is heat sealed to create two small segments, gentle pulling of the tubing (where the two segments meet) separates the tubing and the bags while maintaining a closed system (Fig. 6.3). Bags for cell culture are available in different sizes ranging for as small as 7 mL to 5 L.

As cells in culture proliferate media must often be changed and the culture volume expanded. For cells cultured in up to 600 mL bags, media can be changed by simply centrifuging the bags in floor model centrifuges specifically designed for processing whole blood. After centrifugation the cell-free supernatant can be expressed through the bag's tubing into another bag that has been sterilely connected to the culture bag (Fig. 6.4). After the bag containing the cell-free supernatant is heat sealed and removed, another bag containing fresh media is connected to the culture bag and the media is added to the cell pellet. Alternatively, as cells proliferate they can be transferred into progressively larger bags and fresh media can be added. For cultures requiring 3 or more liters of volume, the contents of the culture bags can be spilt into multiple bags. Bag cultures have been used to expand TIL cultures up to 60 L. When multiple bags are used, at the end of the culture period, the bags can be sterilely linked and the contents can be combined, concentrated and washed using automated closed system instruments as described below.

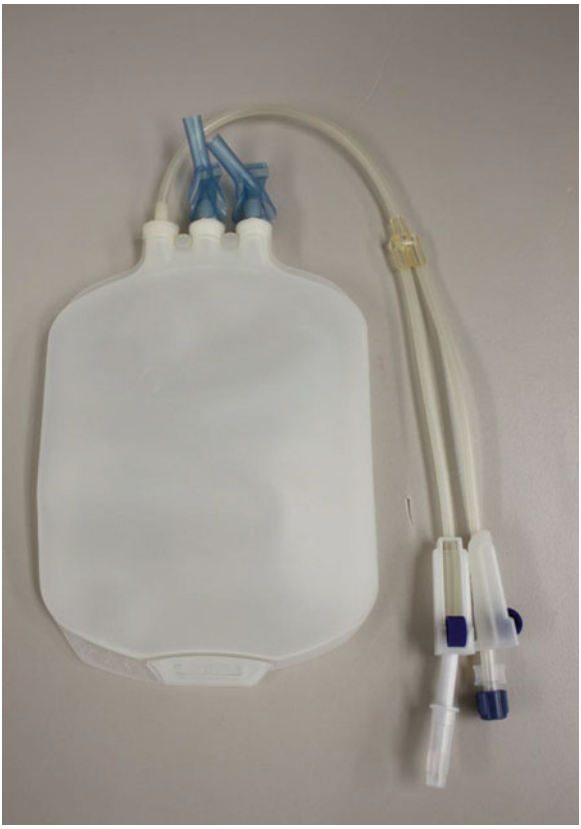

Fig. 6.1 Bag specifically designed for cell culture. This cell culture bag (Lifecell Tissue Culture Flask, Baxter Healthcare Corporation, Deerfield, IL) is made with gas permeable plastic and has two tubing leads which can be connected to other bags or containers using the ports or by sterile connection of the tubing to the tubing of another bag or vessel

One limitation of bag culture is that bags do not lend themselves to automated filling and drainage. Other closed system devices and reactors are available for T cell culture and they will be discussed later in this chapter.

Many automated devices that are used to collect and process blood cells also make use of sterile disposable plastic liners to maintain a closed system during processing. Bags are integrated into these systems that are used to hold the collected cells or solutions for washing. These disposable kits, which are used only once, are sterilized during the manufacturing process and they prevent the cellular product from becoming contaminated microbes and with cells from other donors whose cells have been processed using the same instrument.

Donor Screening and Testing

The first step in the production of cellular therapies is to identify and evaluate the person who will donate the cellular starting material. Donors of cellular therapy products are screened and tested, much like blood donors, to determine if they have

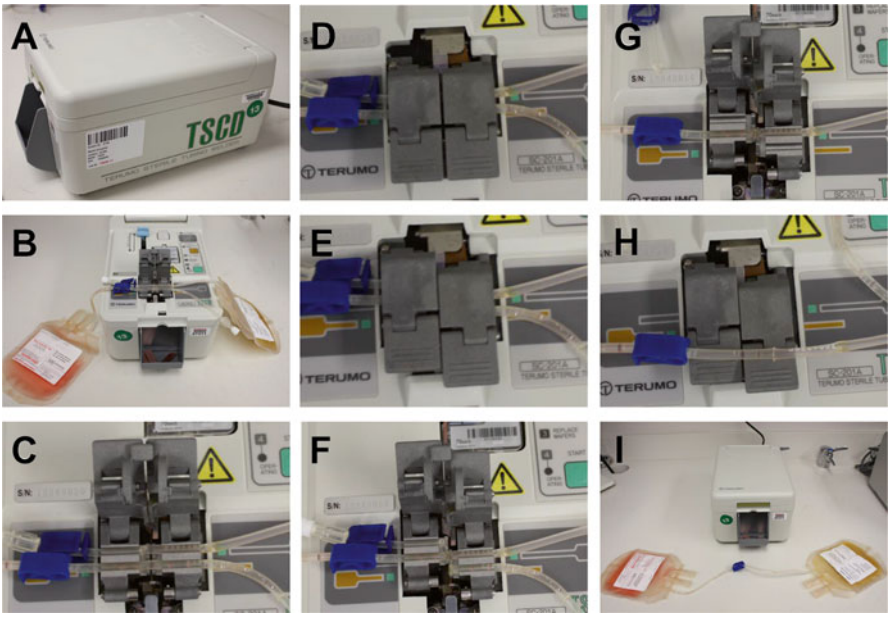

Fig. 6.2 Sterile connection of tubes. This sterile tubing welder (Terumo BCT, Lakewood, CO, USA) (**A**) is used to sterilely connect two separate tubes. It is used to sterilely link bags containing media, cells, reagents or wash solutions. To connect tubing from two bags the instrument's clamps are opened and the tubing to be welded are inserted into the clamps (**B** and **C**). To hold the tubing is place, the clamps are closed and the tubes are welded (**D** and **E**). The connected tubes (**F**, **G** and **H**) and bags (**I**) are shown

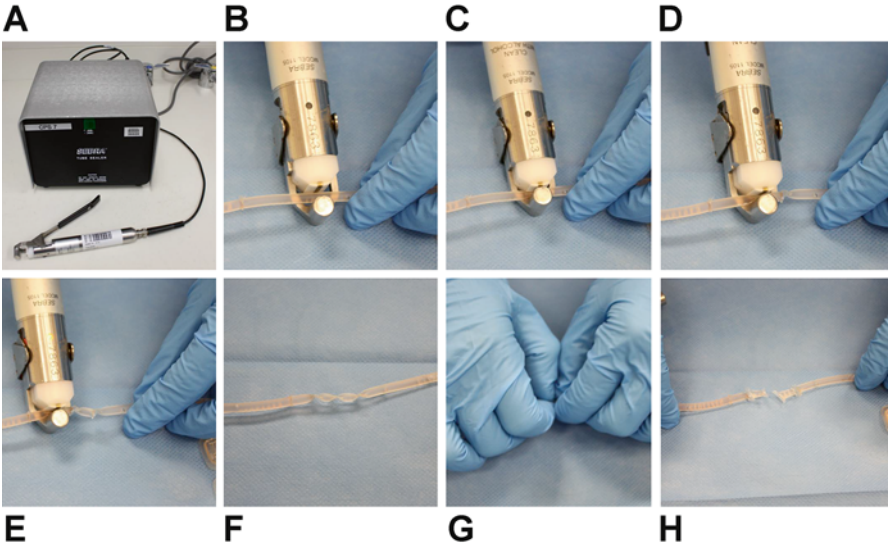

Fig. 6.3 Sealing and separation of tubing. Instruments have been specifically designed to seal tubing and separate culture bags connected by tubing (**A**). The tube is first sealed at the site where the tubing and bags are to be separated (**B** and **C**). After the first seal has been completed a second (**D**) and third seal (**E**) are made within a few centimeters of the first seal (**F**). At the site of the middle seal gentle pressure is used to pull the tubes apart (**G**). The tubes remain sealed at each end and the addition seal in each tube is to ensure that the cultures are not contaminated by a leak at the site of separation (**H**). A Sebra tube sealing device (Haemonetics Corporation, Braintree, MA) is shown

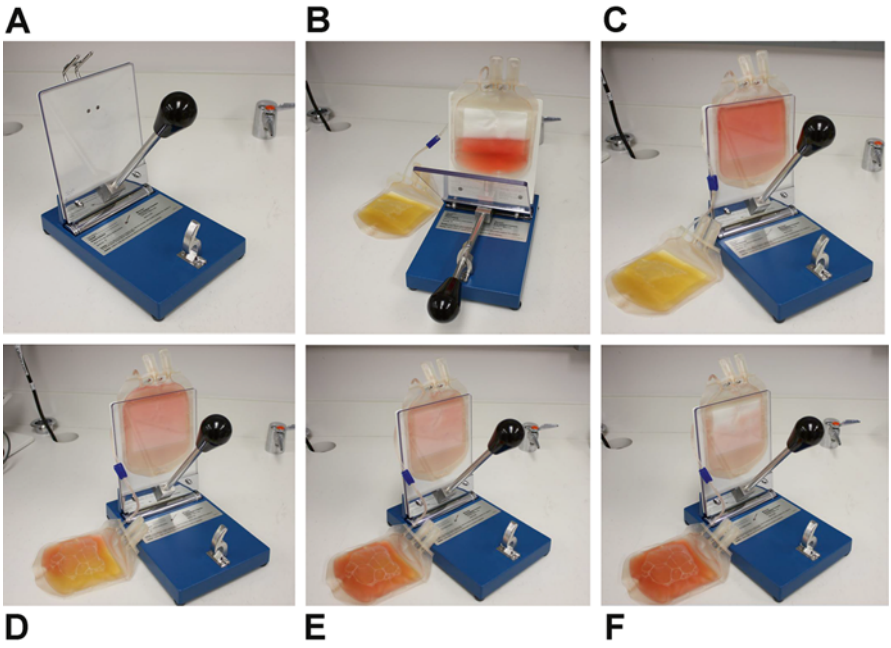

Fig. 6.4 Expression of supernatant from one bag to another. Plasma extractors can be used to separate supernatant from cells that have been pelleted in the bottom of a bag by centrifugation (**A**). The bag with the pelleted cells is placed in the plasma extractor (Fenwal Inc., Lake Zurich, IL, USA) (**B**). The clear spring-loaded plate is released and allowed to slowly force the supernatant from the bag. The speed of remove is controlled using a clamp on the bag's tubing (**C**). The supernatant gradually flows into a waste bag (**D**, **E** and **F**). At the end of the process the cells are resuspended and new media is added to the culture bag. The bags have colored media to better visual the bags

a history of past behavior and experiences that may have exposed them to an infectious agent that could be transmitted by blood cells. Typically, potential donors are screened by asking them a series of well-defined questions that are designed to determine if they have risk factors associated exposure to HIV, hepatitis B, hepatitis C and other infectious agents. The content of these questions are outlined by standards for cell and gene therapies prepared by some professional societies including the AABB (formally the American Association of Blood Banks). Blood from the potential donors is also tested for anti-HIV, anti-HTLV1/2, anti-HCV, HBsAg and Syphilis. Donors of leukocyte rich products such as T cell therapies are also tested for anti-CMV. This screening and testing process is not required when the product collected will be used for autologous therapy, but many centers screen and test autologous donors since the processing of products from patients with infectious diseases may require special precautions to prevent the cross contamination of products intended for other patients that are being processed at the same time. In addition, after the processing of an infectious product is complete, the facility may need more rigorous cleaning to prevent the contamination of products subsequently processed in the laboratory.

Collecting the Cellular Starting Material

For the production of genetically engineered autologous T cell therapies the T cell rich starting material is collected by apheresis using a blood cell separator. Blood cell separators were originally designed to collect neutrophils for transfusion and are now most commonly used to collect platelets for transfusion and peripheral blood mononuclear cell (PBMC) products for cellular and gene therapy.

Blood cell separators can collect large quantities of PBMCs quickly and sterilely. Enough cells for an entire autologous T cell therapy, 5 to 10×10^9 cells, can be collected in a couple of hours. Most of the red blood cells and plasma is returned back to the donor during the process. The blood cell separators make use of one time use sterile disposable kits in which blood is collected from the donor's peripheral vein in one arm and is directed through the instrument where cells and plasma are separated. The blood cell separator collects the desired cell type in to a bag and the cells and plasma that are not needed are returned to a peripheral vein in the donor's other arm. The closed disposable collection system ensures that the products are free from microbial contamination. There is a very small risk that the cells become contaminated during the collection procedure. When blood cell separators are used for the collection of platelet products for transfusion it has been found that only about 1 in 5000 products are contaminated with bacteria [18]. The contamination events are typically due to bacteria from the donor's skin or donor bacteremia.

The most frequently used blood cell separators make use of differences in density to separate cells; consequently, when the instruments are set up to collect lymphocytes, they also collect monocytes which are of a similar density. The lymphocyte-rich PBMC products collected by apheresis are also contaminated with small quantities of granulocytes, red blood cells and platelets. Blood cells separators are very reliable, but the composition of the final PBMC product may vary among donors. The quantities of T cells collected as well as the quantities of contaminating cells can vary. Since the composition of the cells is highly dependent on the donor's blood counts, the composition of products collected from autologous donors who have received prior therapies can be especially variable.

For many T cell therapies the starting material is autologous mononuclear cells collected by apheresis. T cells may then be enriched by elutriation or purified by selection or simply by stimulating T cells which may outgrow other cells in culture. For TIL therapy the starting material is obtained from surgically resected metastatic lesions. TIL can be obtained from small pieces for tumors or from tumors digested with proteolytic enzymes. The use of tumor pieces rather than digests avoids the need to obtain GMP grade enzymes. Traditionally, TIL are obtained for the tumor pieces or digests by culture in plates with IL-2 over several weeks. However, these tissue culture plates are open to the environment and are subject to contamination with bacteria and fungus. It has recently been shown that initial TIL culture can be performed in G-Rex10 gas permeable flasks (Wilson Wolf Manufacturing, New Brighton, MN, USA) at higher cell concentration than classical T flasks or culture bags (Fig. 6.5) [19]. Culture in G-Rex flasks is less susceptible to contamination since these flasks allow T cells to grow at higher densities therefore fewer manipulation are required.

Fig. 6.5 Gas permeable flasks that have been used for T cell culture. These flasks have a gas permeable member at the bottom (G-Rex, Wilson Wolf Manufacturing, New Brighton, MN, USA). Three different size flasks are shown. The smallest flask, G-Rex10, has a gas permeable membrane of 10 cm^2 and a capacity of 40 mL. The middle and largest size flasks both have membranes of 100 cm^2; the middle flask, G-Rex100, has a capacity of 500 mL and the largest flask, G-Rex100L, has a capacity of 2000 mL

Product Segregation

It is important to take measures to ensure that products from different patients are not mixed up or cross contaminated. The vessels containing the cells are, of course, labeled with the recipient's name and hospital number, but specific laboratory practices should be in place to reduce the possibility of misidentifying products. Products manufactured for different patients should be keep separate and laboratory staff should work on products from only one patient. If products from multiple patients are to be processed simultaneously, each product should be processed in a separate biosafety cabinet, they should be cultured in a separate incubator and by separate staff. Strict control of product labels is useful in preventing mislabeling of products. At receipt of the starting material, release of the product from inventory and at other critical points, two processing staff should confirm that the appropriate product has been received, issued or selected for the next processing step or operation.

Cell Isolation

For many cell manufacturing protocols contaminating cells must be removed from PBMC products collected by apheresis before cell culture or further manufacturing can begin. Most often red blood cells must be removed. For some manufacturing protocols highly enriched T cells must be obtained from the starting PBMC product. A variety of methods can be used for RBC removal and lymphocyte enrichment. To obtain highly enriched populations of T cells selection processes using monoclonal antibodies must be used.

Removal of Contaminating RBCs

Several methods are available to remove RBCs. If large quantities of RBCs are present in the product and if some leukocyte loss can be tolerated, the product can be centrifuged and the leukocyte rich buffy coat can be isolated. Up to 80 % of RBCs can be removed by isolating the buffy coat with a loss of less than 20 % of the leukocytes. For products of approximately 250–500 mL total volume, the product can be placed in a bag and centrifuged in a floor model centrifuge to isolate the buffy coat. For products of larger volume, automated instruments made for cell washing such as the Cobe 2991 cell processor (Terumo BCT, Lakewood, CO, USA) can be used to isolate buffy coats. The Cobe 2991 is automated, but less so than other instruments [20, 21].

Another simple method to remove RBCs involves sedimentation. When 6 % hetastarch in 0.9 % sodium chloride, an infusible grade solution that is used for volume expansion, is added to a cell suspension containing RBCs, the RBCs form rouleaux and settle quickly. After a volume of hetastarch equal to approximately 25 % of the final product volume is added to the RBC-containing product and the suspension is mixed, the RBCs settle over 30–90 min and the leukocyte rich supernatant can be removed. RBC sedimentation with hetastarch is simple, but some leukocytes are lost with the RBCs. RBC sedimentation with hetastarch works well for products that are up to 400 mL in volume. The sedimentation process can be performed in a 600 mL bag and devices designed to express plasma from one unit of centrifuged whole blood (Fig. 6.2) can be used to express the leukocyte rich supernatant into a culture bag.

Ammonium chloride lysis is another method for RBC removal. Sterile solutions for RBC lysis such as ACK Lysing Buffer (Lonza, Allendale, NJ) are commercially available. The PBMCs are incubated with a solution of ammonium chloride until RBC lysis is complete, but the time of incubation should not exceed 10 min. The cells are washed to remove free hemoglobin and ammonium chloride at the conclusion of the process. Lysis allows for greater removal of RBC without loss of leukocytes. This reagent is not approved specifically for human use, but it is used by some clinical laboratories safely as an ancillary reagent, i.e. used in production, but not infused.

Density gradient sedimentation can also be used to separate lymphocytes from RBCs. Density gradient separation has an advantage over other methods in that it not

only removes RBCs but it separates the lymphocyte-rich mononuclear cells from granulocytes. This method involves centrifuging the cellular product over ficoll. During centrifugation the lymphocytes and monocytes remain at the top of the ficoll gradient, while the granulocytes and RBCs go to the bottom of the gradient. For small volume products ficoll separation can be performed in 50 mL conical tubes. For larger volume products, ficoll density gradient separation can be performed in bags, blood cell separators or cells washers such as the Cobe 2991 [22]. Another instrument that is available for ficoll density gradient separation is the Sepax 2 (Biosafe SA, Eysins, Switzerland). This instrument has been specifically designed to remove plasma and RBCs from umbilical cord blood components or isolate mononuclear cells from marrow aspirates. It can also be used to isolate buffy coats from PBMCs. One advantage of this instrument is that it is highly automated and provides documentation of the RBC depletion process for the product processing record.

Cell Enrichment

For the production of some T cell therapies it is desirable to use highly enriched T cell, B cell or T cell subset populations as the starting material. For many of these protocols, PBMCs products collected by apheresis are the starting material. The PBMC products can either be enriched or depleted of specific cell populations using commercially available GMP quality monoclonal antibodies conjugated magnetic beads. Antibodies conjugated to magnetic beads are incubated with the PBMCs product and the bound cells are removed with an external magnet. Commercial GMP magnetic beads conjugated to antibodies with many different specificities including CD3, CD4, CD8, CD14, CD19, CD25, CD34, CD56 and others are available from Miltenyi Biotech, Bergisch Gladback, Germany [23–27]. Miltenyi also manufactures the CliniMACS which is an instrument fitted with sterile plastic closed system disposables that can used to isolate cells from clinical PBMC apheresis products. This system can be used for positive selection to isolate a specific cell type such as CD3 or CD4 cells or negative selection to deplete a PBMC product of a specific cell type. When used for positive selection a final product that contains approximately 90 % of the desired type of cells can usually be obtained and typically, approximately 70 % of the desired cell type can be recovered. With this system of monocloncal antibodies and paramagnetic beads multiple negative cell selections, but only one positive cell selection can be performed at one time due to the high affinity of the antibodies and the permanent nature of the conjugation to the magnetic beads. While the CliniMACS system is semi-automated, a considerable amount of labor is involved. A new system has recently been developed that allows for the more automated separation of cells, the Prodigy. This system can also be used for cell incubation and washing, however, it has not yet been adapted for T cell applications.

Another system that makes use of magnetic beads uses monoclonal antibodies bound to *Strep*tamers@ which are conjugated to magnetic beads using a streptavidin derivative, Strep-Tactin@ (IBA GmbH). After the cells are selected, they can be

released from the beads using a biotin solution. When the cells are released, the antibody *strep*tamer complexes break up and the low affinity antibodies are released from the cells. This ability to separate the cells, antibodies and the magnetic beads allows for multiple positive selections.

Cell Culture

Culture Vessels

Classically lymphocytes have been cultured in T flasks. However, T flask culture has some limitations because they are open systems. Culturing cells in T flasks is especially problematic when large quantities of cells must be grown. In order to culture large quantities of cells, the number of flasks used must be increased which increases the possibility of microbial contamination. When large quantities of T cells must be produced they are usually grown in multiple culture bags. Culturing of cells in bags is now common for phase I and phase II manufacturing of T cell products. However, bag cultures have some limitations, one being that cells can only be grown to a concentration of approximately $2.0–3.0 \times 10^6$ cells per mL. Growing cells at a higher concentration has the advantage of requiring less cytokines, less media and less media additives reducing the cost of the culture. In addition, smaller culture volumes make washing and concentrating the cells easier. Smaller volume cultures also require less labor for feeding, media changes, cell concentration and cell washing and more instruments are available for washing and concentrating smaller volumes.

An alternative to bag culture which allows T cells to be grown at greater concentrations are bioreactors. Closed system hollow-fiber cartridge [28] or circular chamber [29] bioreactors have been developed and have been demonstrated to support T cell growth, but they have limited by their lack of scalability. Typically they are automated, but have a very limited availability of cartridge sizes. Scaling up the production of cells generally requires growing cells in more than one bioreactor which increases the complexity and cost of the process. In addition, these bioreactors require the purchase of expensive hardware. The validation and maintenance of the hardware can also be time consuming and expensive. The disposable hollow fiber and circular cartridges can also be costly. One bioreactor, the wave, uses bags rather than cartridges and can grow cells in a wide range of bag sizes, making it very scalable. The wave gentle rocks the culture bag while gradually adding media and oxygen and it has been used to expand TIL [30]. While it is effective, it still requires capital investment in equipment, validation of the system and staff training.

Another alternative to bag culture is the growth of T cells in gas permeable flasks. Flasks with gas permeable membrane bottoms, G-Rex flasks (Fig. 6.5), allow T cells to be grown up to approximately 1×10^7 cells per mL. These gas permeable flasks have been used to grow TIL [3, 19] and viral specific cytotoxic T cells [31]. A number of modifications have been made to the flasks to facilitate GMP cell manufacturing. Caps for the flasks are available that include tubes that allow for a

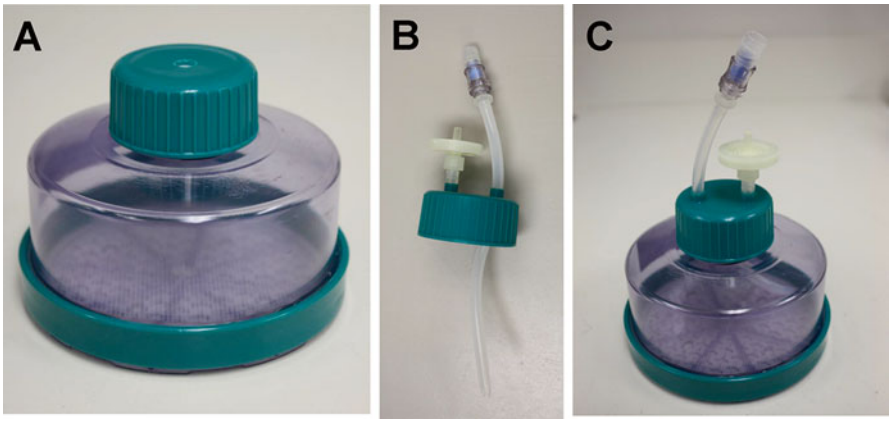

Fig. 6.6 Gas permeable flask and cap with tubes for closed system media addition and removal. Gas permeable flasks (G-Rex, Wilson Wolf Manufacturing, New Brighton, MN, USA) with 500 mL capacity and 100 cm^2 gas permeable membrane, G-Rex100 (**A**) are available with a cap that contains a tube and a vent that allow filling or emptying of the flask without opening the cap (**B**). This creates a more closed system that reduces the risk of microbial contamination (**C**)

more closed system and for easier filling and emptying of the flasks (Fig. 6.6). An instrument has recently become available that allow the automated sterile removal and addition of fluid and sterile cell harvesting (Fig. 6.7). Removing fluid from the gas permeable flasks with a repeater pump is problematic because the vacuum generated in the flask by the pump causes the flexible gas permeable membrane on the bottom of the flask to rise and could rupture the membrane. The device used to remove fluid from gas permeable flasks pushes sterile air into the flasks to displace fluid through tubing and into a bag(s). One limitation of these flasks is that they are only available in sizes that hold 40 to 2000 mL. However, flasks are being developed that will hold 4–5 L of media.

T Cell Expansion

Several media have been designed specifically for T cell culture. However, to support T cell expansion these media must be supplemented with human serum. Group AB serum is used since it lacks anti-A and anti-B which could lyse RBCs present in the cultured cells from blood group A, B or AB subjects or react with group A or B antigens adsorbed by other cells. Most T cell expansion protocols also involve the culture of the cells with a cytokine that acts as a growth factor; IL-2 is most commonly used. While IL-2 promotes T cells proliferation, it also induces T cell maturation. In addition, the culture of antigen- or anti-CD3-stimulated T cells in the presence of IL-2 induces an increase in the number of T cells expressing FOXP3 which are know as T regulatory cells (Treg) [32]. Treg cells have a potent immune

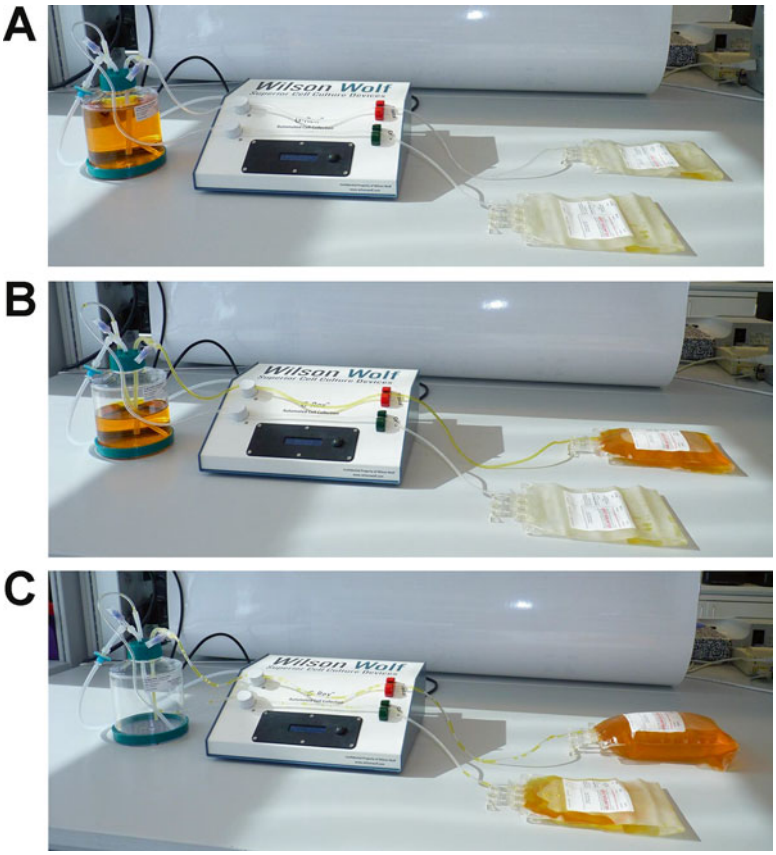

Fig. 6.7 Closed system removal of media from gas permeable flasks. Removing media from the closed system gas permeable flasks using negative pressure generated with a syringe or repeater pump can cause displacement of the flexible gas permeable membrane off the bottom of the flask which could damage the membrane. The automated cell recovery device (Wilson Wolf Manufacturing) displaces fluid from the flasks using positive pressure (**A**). Sterile filtered air displaces media without displacing the gas permeable membrane (**B** and **C**)

suppressive function and can inhibit in vivo the effects of adoptively transferred T cells [33]. Other cytokines are being tested for T cells culture that do not expand regulatory T cells and which promote a different and possibly more potent T cell phenotype. For example the culture of cytotoxic T cells in IL-21 increases the quantity of antigen-specific cytotoxic lymphocytes (CTLs) in culture while reducing the quantity of FOXP3 expressing suppressor cells [34, 35]. In addition IL-21 produces more CTLs with a central memory phenotype [36]. IL-7 and IL-15 can also be used for T cell expansion. The culture of T cells in IL-7 does not increase the number of Tregs, however, culture in IL-15 does [32, 33]. Culture of naïve T cells in IL-7 plus IL-15 allows the cells to maintain a memory T cell phenotype [37].

Antibodies, antibodies conjugated to beads and feeder cells are often added to T cell cultures in order to increase proliferation and expansion. TIL can be obtained from melanoma digests or fragments by culture in IL-2 alone; this is known as initial TIL culture [38]. However, for the more extensive expansion of isolated TIL required for clinical therapy, a "rapid expansion" protocol is used which involves the culture of TIL with IL-2, antibodies directed to the T cell receptor, anti-CD3, and allogeneic leukocytes as feeder cells [38]. GMP grade IL-2 and anti-CD3 are commercially available, but obtaining feeder cells is more difficult. PBMCs collected by apheresis from healthy subjects are used by some groups as feeder cells for rapid expansion of TIL. These cells are gamma irradiated to a sufficient degree to prevent their proliferation in vitro and in vivo. PBMCs pooled from several healthy subjects are typically used. The disadvantage of using PBMCs is that a mechanism must be in place to collect PBMCs from healthy subjects by apheresis and the donors must undergo health history screening and be tested for markers of infectious agents. As a result this process is expensive and not available to all centers.

There are some alternatives to the use of PBMCs feeder cells for T cell expansion. Magnetic beads conjugated with anti-CD3 and an antibody to the co-stimulatory molecule CD28, have also been found to effectively stimulate T cell growth in the presence of IL-2 [39, 40]. GMP grade beads conjugated to anti-CD3 and anti-CD28 are available [40]. These beads are magnetic which allows for their removal with a magnet external to the culture vessel at the end of the culture period (Fig. 6.8). While these anti-CD3/anti-CD28 beads are being use for the GMP manufacture of some T cell products, some groups have found that they preferentially induce the expansion of CD4+ T cells over CD8+ T cells [41].

Another alternative to PBMCs as feeder cells for T cell expansion are artificial antigen presenting cells (APCs) that are made from the leukemia cell line, K562 cells. The K562 cells that are genetically engineered to express Fc receptors and costimulatory ligands, such as 4-1BBL [42]. The Fc receptors are loaded with anti-CD3 and anti-CD28. The presence costimulatory ligands along with anti-CD3 and anti-CD28 allow these cells to function as APCs and support the expansion of T cells [43]. These artificial APCs have been used to expand TIL from melanoma and ovarian cancer. The degree of expansion and cell characteristic are similar to those expanded with allogeneic PBMCs but at lower TIL:APC ratios, meaning fewer feeder cells are required. In addition, these artificial APCs maintained a favorable CD8/CD4 ratio and FOXP3+ CD4+ cell frequency. Artificial APCs represent a more standardized "off-the-shelf" cellular platform for TIL and T cell expansion [43].

Genetically Engineering T Cells

Many clinical T cell therapy clinical protocols now involve the genetic engineering of T cells in order to express high affinity T cell receptors specific for tumor antigens or CAR specific for tumor antigens. Many of these studies make

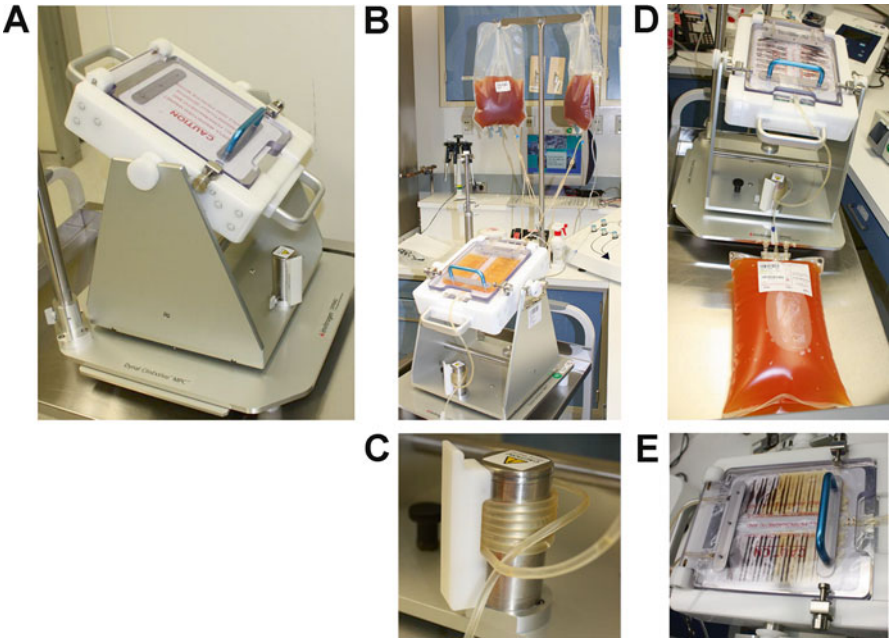

Fig. 6.8 Removal from cultured T cells of magnetic beads conjugated with CD3 and CD28 antibodies. A device (Dynal ClinExVivo MCP, Life Technologies AS, Oslo, Norway) with two magnets is used to remove magnetic beads from the cultured cells (**A**). The primary magnet is encased in the white plastic platform. A smaller secondary cylindrical secondary magnetic is below the primary magnetic. T cells cultured in bags with anti-CD3/CD28 magnetic beads flow through a bag held against the large primary magnet with a clear plastic plate (**B**), and into a collection bag (**C**) through tubing wrapped around the secondary magnetic (**D**). Most of the anti-CD3/CD28 beads are removed by the primary magnetic (**E**)

use of retroviral vector and transduction is carried out in open vessels. Typically, T flasks or 6-well plates are coated with retronectin, vector is added and the flasks are incubated and then centrifuged. After the supernatant is removed the T cells are added to the retroviral vector preloaded plates [44]. Recently, simpler, closed system methods for the transduction of T cells with retroviral vectors encoding anti-CD19 CAR have been developed [45, 46]. The two closed system methods involve the transduction and culture of T cells in culture bags. The bags are coated with retronectin and vector is added. One method involves the centrifugation of the bags containing the vector and T cells, spinoculation [46], while another simply adds the T cells to retronectin coated bags that have incubated for 2 h with the vector [45]. Both of these methods result in similar transduction efficiency to that obtained with multi-well plates and both are being used in clinical trials.

Concentrating and Washing

At the end of the culture period cellular therapies must be concentrated and washed. Often the culture media contains antibodies, cytokines and growth factors in quantities that are too great to be safely administered with the cellular therapy and the final product must be washed before it is given to the recipient. Typically, after washing, the cells are resuspended in an infusible fluid such as saline or Plasma-Lyte A (Baxter Healthcare Corporation, Deerfield, IL, USA) supplemented with human serum albumin. In addition, the final volume of the cells in culture is often several liters or greater, but the ideal volume for cellular therapies that are given intravenously is only 100–1000 mL. Consequently, the volume of the product must often be reduced. For products whose final volume is a liter or less the product can easily be concentrated and washed using bags and a floor model centrifuge. For products whose volume is more than 1–1.5 L it is desirable to use a more automated system for washing and concentrating the cells. Automated systems allow for the more rapid concentration and washing of large volume cultures. Rapid washing and concentration is important because of the limited stability of the T cell products suspended at high concentration in infusible media. Blood cell processors such as the Cobe 2991 cell processor (Terumo BCT) have been used for this application. Fenwal, is working to develop a new spinning membrane cell washer. The Cobe 2991 processes cells discontinuously (Fig. 6.9). It can concentrate up to 600 mL in one concentration/wash cycle and each cycle requires approximately 10 min to complete. The size of product that can be concentrated and washed using a Cobe 2991 over 2–4 h is limited to 5–10 L. Some laboratories are using instruments designed to recover autologous blood during surgery for washing cellular products. In addition some companies are working on instruments that make use of tangential flow to concentrate and wash cellular products.

Product Testing

It's important to evaluate the cellular product at several time points during the manufacturing process. The starting material is tested to be sure that a sufficient quantity of the desired cells are present and to ensure that quantities of contaminating cells are below critical levels. The starting material is usually tested for sterility and identity. The final product should be tested for quantities of desired and contaminating cells, endotoxin level and sterility. For cultured products, in addition to sterility, mycoplasma and endotoxin testing is required. It is also desired to measure the critical biological function or potency of the final product.

Conclusions

Many procedures have been described that allow for the manufacturing of high quality cellular therapies to support phase I/II clinical trials. Instruments, devices and reagents are available which help improve the manufacturing process. The growing

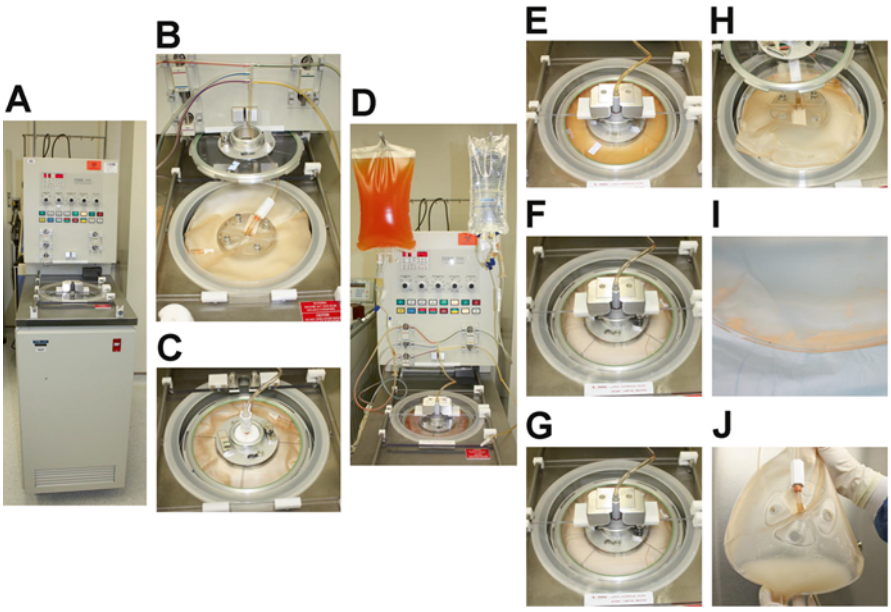

Fig. 6.9 An automated cell washing device and it's sterile, closed, one-use, plastic liner. The Cobe 2991 cell processer (Terumo BMT, Lakewood, CO, USA) can be used to wash cultured T cells (**A**). The sterile plastic circular disposable liner is placed into the instrument (**B**) and the clear cover is locked into place (**C**). Cultured cells are loaded into the circular plastic container as the container spins (**D** and **E**). Approximately 600 mL of cultured cells are added. After approximately 6 min of centrifugation, the supernatant is removed and the pelleted cells are resuspended by agitation (**F**). The process of adding 600 mL of the cultured cells, centrifugation, supernatant removal and resuspension continues until all the cultured cells have been concentrated. Each cycle takes about 10 min. The pelleted cells are then washed by using several cycles of resuspension in 600 mL of saline, centrifugation and supernatant removal (**G**). The cells are then washed with the solution that will be used for infusion or for cryopreservation. After the washes are complete the pelleted cells (**H** and **I**) are resuspended in infusion or cryopreservation solution (**J**)

success of T cell therapies has resulted in investment by academic investigators and industry in methods to further improve the manufacturing processes by creating new reagents and devices. Combining these procedures, instrument and reagents in novel ways is allowing for the rapid development methods for manufacture of emerging cell therapies that are sterile, consistent and potent.

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