

**Division of Cellular and Gene Therapies
Office of Cellular, Tissue, and Gene Therapies
Center for Biologics Evaluation and Research
Food and Drug Administration**

**Cellular, Tissue and Gene Therapies Advisory Committee Meeting
March 29, 2007**

CMC Briefing Document

PROVENGE® (Sipuleucel-T)
For the treatment of men with asymptomatic metastatic androgen independent prostate cancer

Table of Contents

INTRODUCTION	3
<hr/>	
MANUFACTURING PROCESS	4
<hr/>	
MANUFACTURING PROCESS OVERVIEW	4
CELL SOURCE	5
INCUBATION WITH PA2024	5
HARVEST, WASH, FORMULATION	6
LOT RELEASE	6
PRODUCT VARIABILITY	7
<hr/>	
CELLULAR COMPOSITION	7
CELL NUMBER	9
PRODUCT POTENCY	11
<hr/>	
CD54 UPREGULATION DURING IN VITRO CULTURE	11
ANTIGEN PRESENTATION BY CD54 EXPRESSING CELLS	15
CD54 UPREGULATION AND CD54 CELL NUMBERS AS A MEASURE OF POTENCY	19
REFERENCES	21
<hr/>	

List of Figures

Figure 1. Sipuleucel T- Manufacturing Scheme.....	4
Figure 2. Depiction of PA2024 fusion protein.....	6
Figure 3. Proportion of major cell populations in the final product for autologous vaccination into patients.....	8
Figure 4. Percentage of leukocyte populations at various manufacturing stages of sipuleucel-T.....	9
Figure 5. Leukocyte total cell numbers present within the starting leukapheresis material, processed cells, and the final product for autologous vaccination into patients.	10
Figure 6. Upregulation of cell surface markers after culture with PA2024.....	12
Figure 7. The effect of GM-CSF on CD54 upregulation.....	13
Figure 8. Expression of cell surface markers in the CD54 positive cell population before and after <i>ex-vivo</i> culture with PA2024.....	14
Figure 9. Correlation of Immune Activation-Associated Markers with PA2024-FITC Uptake.....	15
Figure 10. Correlation of Lineage Markers with PA2024-FITC Uptake.....	16
Figure 11. Antigen presentation to PAP-specific T cell hybridomas resides in the CD54 cell population.....	17
Figure 12. PAP antigen presentation by other cell types.....	18
Figure 13. Effect of GM-CSF on PAP Antigen Presentation.....	19
Figure 14. CD54 cell number and CD54 upregulation present in the final product.....	20

List of Tables

Table 1. Enrichment of APCs.....	5
----------------------------------	---

INTRODUCTION

Sipuleucel-T (Provenge™, APC8015) is a patient-specific autologous cellular therapy for the treatment of hormone refractory prostate cancer. The active ingredient of the product is antigen presenting cells that present a prostate cancer antigen, prostatic acid phosphatase, to the immune system. The prostatic acid phosphatase (PAP) is delivered to the antigen presenting cells fused to an immunostimulatory factor, granulocyte macrophage colony stimulating factor (GM-CSF). This fusion protein, PAP-GM-CSF, is referred to as PA2024. The antigen presenting cells are manufactured using cells collected by apheresis from the patient which are incubated with recombinant fusion protein PA2024 for 36-44 hours.

The proposed mechanism of action of sipuleucel-T is that of an active immunotherapy to generate a prostate cancer specific immune response that will fight the patient's cancer. The cells that comprise sipuleucel-T are not intended to have a direct cytotoxic effect. Instead, the anti-tumor effect is generated by the presentation of the PAP antigen by activated antigen presenting cells present in the population of cells contained in sipuleucel-T to immune cells in the body such as T cells which will then mount an immune attack against the prostate tumor. Thus, this product relies on the patient's immune system to develop a specific response that can then kill the tumor. This mechanism of action is unlike chemotherapeutic drugs that directly kill the tumor cells. It is also different from several other immunotherapies which either generically stimulate the immune response, such as interleukin-2 (IL-2), or specifically target the tumor, such as herceptin. Because the product requires the development of an immune response after administration, there is a delay in the potential effect of the product; the generation of the immune response can take up to several weeks.

To manufacture sipuleucel-T, a patient's cells are collected by apheresis. Red blood cells and granulocytes are removed from the apheresis product by two buoyant density gradient separations, retaining the populations of leukocytes. PA2024, which consists of the prostatic acid phosphatase (PAP) linked to GM-CSF, is then added to the cells. The GM-CSF portion of the protein helps to target the PAP protein to antigen presenting cells and activate those cells. GM-CSF is a multi-lineage colony stimulating factor that principally affects the proliferation, differentiation, and activation of granulocytes and macrophages (Nguyen et al., 2002). The PAP provides the tumor specific antigen that will direct the immune system to target prostate cancer. PAP is a protein that is highly expressed by prostate and prostate carcinoma cells and only expressed at very low levels in a few other tissues (Cunha et al., 2006).

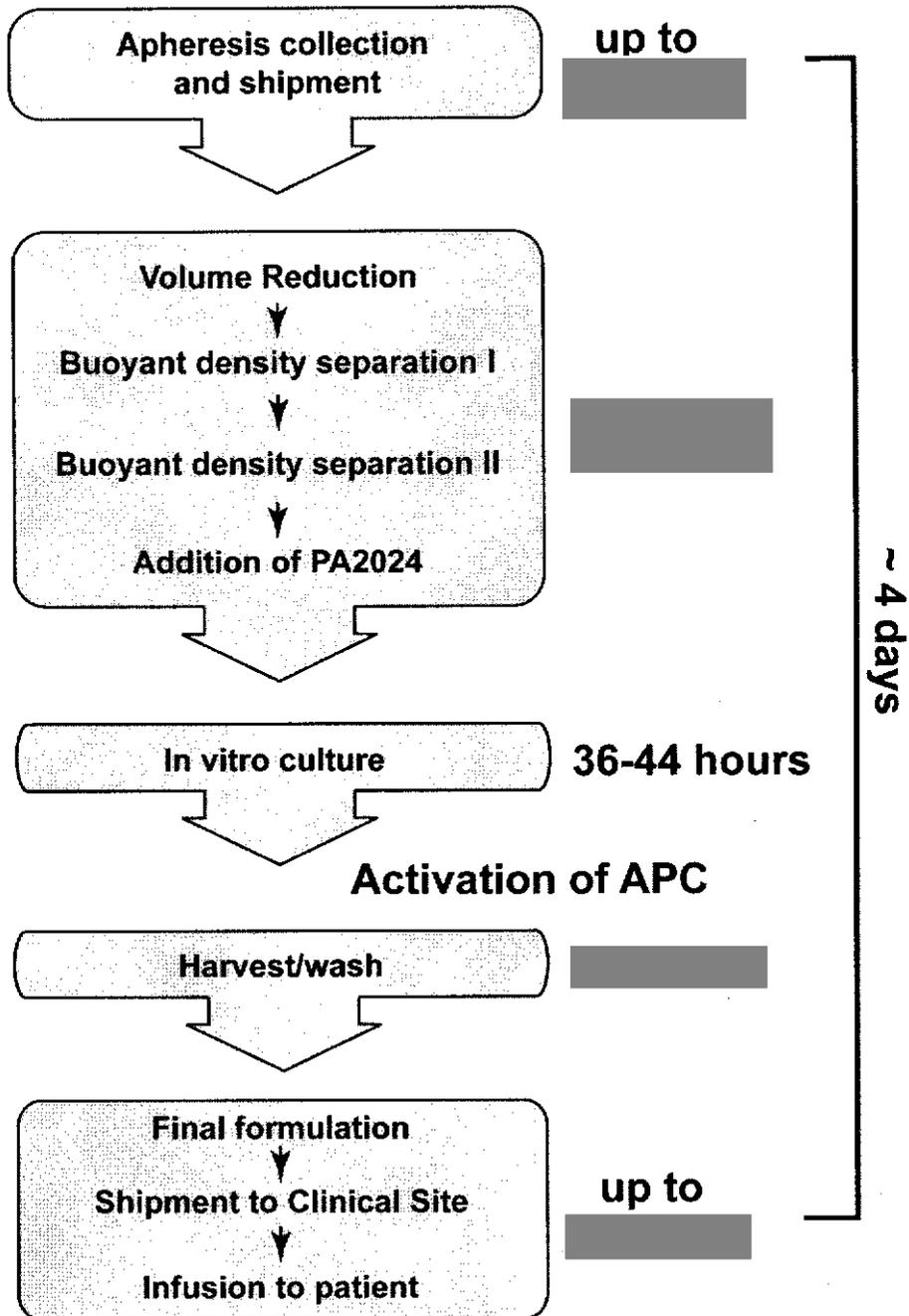
The course of therapy is 3 doses, given at approximately 2 week intervals. Each apheresis produces one dose of product, therefore the patient undergoes 3 separate apheresis procedures. Each apheresis product goes through the identical manufacturing process and is considered a unique lot of product. If a lot of product fails to meet requirements for quality, the patient must undergo an additional apheresis to make a new lot of product. Each dose is shipped and administered fresh (without cryopreservation) within [REDACTED] of manufacture.

MANUFACTURING PROCESS

Manufacturing Process Overview

The manufacturing process for Sipuleucel-T is depicted below:

Figure 1. Sipuleucel T- Manufacturing Scheme



Cell Source

The source of autologous cells for sipuleucel-T is obtained by apheresis of the patient. Antigen presenting cells (APC) are found in different anatomical locations and at different stages of maturity. In whole blood the primary antigen presenting cell is monocytes, that *in vivo* can infiltrate tissues and mature to become macrophages or dendritic cells (Rice et al., 2004).

Two successive buoyant density gradient centrifugation separation steps are performed to remove red blood cells and granulocytes from the leukapheresis. One rationale for performing this step is that granulocytes and red blood cells are not likely to participate in antigen-specific immune responses, and granulocytes by phagocytic activity would likely take up the fusion protein during the subsequent culture making it unavailable for the antigen presenting cells in the product. These gradients are the only enrichment procedure for APC in the manufacturing scheme. The table below (Table 1) demonstrates the ability of this step to reduce the number of red blood cells and granulocytes. These results show that roughly [REDACTED] of the granulocytes are removed by this process.

Table 1. Enrichment of APCs.

	RBC/mL	% Granulocytes	Granulocytes/mL
Apheresis product before processing	[REDACTED]	[REDACTED]	[REDACTED]
Buoyant Density Gradient 1	[REDACTED]	[REDACTED]	[REDACTED]
Buoyant Density Gradient 2	[REDACTED]	[REDACTED]	[REDACTED]

Although monocytes are the major population of APC in the product, they typically make up less than 25% of white blood cells. In addition to monocytes, the final product includes T cells, B cells, and NK cells. It is unclear what, if any, effect T, B, and NK cells have on the biological activity of the product. The applicant has established that the majority of the APC activity is not associated with T and B cells (discussed below).

Incubation with PA2024

The next step in the manufacturing process is the addition of the PA2024 fusion protein and *in vitro* culture for 36-44 hours at 37°C. The purpose of this step is for the APC to take up, process, and present the PAP antigen and become activated to express costimulatory molecules. The APC will bind PA2024 by way of the GM-CSF receptor and internalize the fusion protein, process the PAP antigen portion, and present it in context of class I and class II major histocompatibility complex (MHC). The PAP antigen sequence will contain many potential epitopes that will differ depending on MHC haplotype of the patient. Data were not provided to allow assessment of whether there is any impact of MHC haplotype on the patient's immune response or clinical outcome. A schematic diagram of the PA2024 fusion protein is shown below (Figure 2). GM-CSF is included in the fusion protein to stimulate uptake of the PAP

antigen by and activation of the APC.

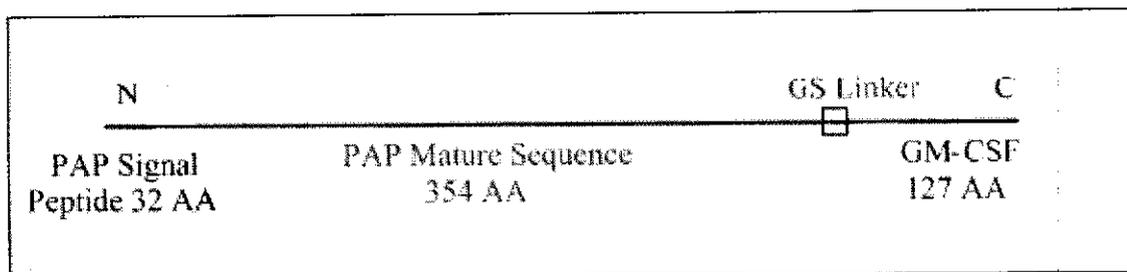


Figure 2. Depiction of PA2024 fusion protein.

PA2024 is composed of PAP antigen, which is fused to full length GM-CSF sequence through a GS peptide linker. GM-CSF will aid in APC stimulation and the PAP sequence will be processed and presented to T cells via MHC class I and class II.

Harvest, Wash, Formulation

Following 36-40 hours of incubation in PA2024 the cultured cells are collected, washed and formulated for shipping. The patient's cells are packaged in a blood bag and shipped at 4-8°C to the infusion site. Lot release testing is performed at the time of formulation. The applicant has established an [redacted] shelf life for the final product; the product is shipped and infused within [redacted] of formulation. Therefore, the assays used for the required testing were designed to have a rapid readout to provide results within hours.

Lot Release

Testing of each lot of biological products for sterility, identity, purity, and potency is a regulatory requirement for licensure (21 CFR 610). Identity, purity, and potency assays are specifically developed for individual biological products to serve as a measure for product quality and consistency. The purpose of identity testing is to adequately identify the product as that designated on the final container and package labels and distinguish it from other products being processed in the same facility. Assessment of purity includes a measurement of pyrogenic substances, and any extraneous material that is unavoidable in the manufacturing process. For cellular products this may include measurement of populations of cells in the final product that are not proposed to be the active cells. Potency is a measure of the relative biological activity of the product and can be measured either in vitro or in vivo. Identity, purity, and potency are critical product characterization parameters that are used not only for lot release, but are also important parts in stability studies and comparability studies (performed after changes to the product or process).

For sipuleucel-T, measurement of identity, purity, and potency included an enzyme linked immunosorbent assay (ELISA) for the presence of the PAP antigen, total cell viability, and the total number of CD54 positive cells and the upregulation of CD5 expression (fold increase that results from culture in presence of PA2024). The sipuleucel-T product dose is based on the total number of CD54 positive cells. The following sections discuss some of the data that were collected to characterize the product and to justify their potency assay.

PRODUCT VARIABILITY

As stated above, the manufacturing process for sipuleucel-T reduces red blood cells and granulocytes but otherwise does not significantly alter the relative percentages of the leukocyte cell types present in the apheresis. There is no positive selection of any single cell type and there is no significant expansion or proliferation of any particular cell type. There is both inter-patient variability as well as intra-patient variability between individual patient vaccination lots. This variability includes 1) the relative percentages of the leukocyte cell types, 2) the total number of cells in the final product, and 3) the level of activation of the final product. These three properties are discussed individually below. All vaccine lots are produced by the same established procedures, with only slight differences in incubation and processing times that are within validated process parameters.

Cellular Composition

The final cellular product is a mixed population of leukocytes. The major populations of cells that are included in the final product are those found in normal human blood. They include T cells, B cells, NK cells, and monocytes. During product development, the sponsor measured the relative percentages of these leukocytes at various points in the manufacturing process and in the final product. Broad ranges in percentage of T, B, NK, and monocyte populations were observed in the incoming apheresis, both between patients and between the 3 collections from any individual patient.

For the proposed mechanism of action of sipuleucel-T, CD54 positive APC are the most important cell type present in the product. Though CD54 is a general leukocyte marker, CD54 positive cells as defined by the applicant in their analysis parameters (discussed later in the briefing document), would correspond largely to monocytes, which are CD14 positive cells. Thus the APC would express both CD14 and CD54. In whole blood monocytes typically account for less than 10% of white blood cells, and after leukapheresis approximately 20-30% (Nguyen et al., 2002; Shaz et al., 2006). By comparison, CD3 positive cells account for about 25% of white blood cells and approximately 50% after leukapheresis (Wolf et al., 2005) The actual percentage of monocytes in the leukapheresis and in the final product will vary widely from patient to patient. It is also possible that the immune response generated after receiving a dose of the product may affect the number and percentages of these cells in each patient's subsequent apheresis collections. Figure 3 below depicts the range in the percentage of CD54 positive (APC; predominantly monocytes), CD3 positive (T cells), CD14 positive (monocytes), CD19 positive (B cells) and CD56 positive (NK cells) in the final product based on total nucleated cell number. To help describe and visualize the level of variation between individual patient leukapheresis units and between different lots of the final product, the applicant presented the data in the form of box and whisker plots. Data plotted in this way is described in terms of the median, quartiles of the median, and minimum and maximum quartile extremes, with outliers plotted individually.

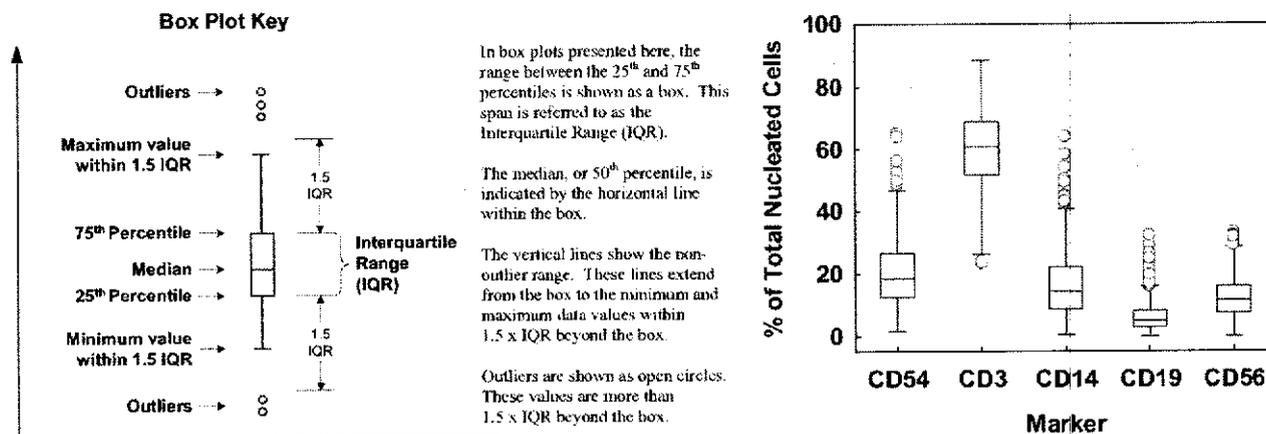


Figure 3. Proportion of major cell populations in the final product for autologous vaccination into patients.

The cellular composition of the final product includes predominantly CD3 positive T cells, CD54 (leukocytes), CD14 (monocytes), CD56 (NK cells), and CD19 (B cells). Although CD54 is present on most leukocytes, it is expressed at different levels in different populations of cells. By the expression criteria defined by the applicant, most of the CD54 positive fraction is accounted for by CD14 positive monocytes. Data is based on 526 lots.

As expected, the predominant cell type present in the starting material and final product is CD3 positive T cells. The next most prevalent population is monocytes (CD14 positive) cells. Monocytes represent the major population of APC present in peripheral blood and this explains why CD14 positive and CD54 positive percentages are nearly equivalent. CD19 positive B cells and CD56 positive NK cells make up smaller proportions of the final product.

The impact of each of the major steps in the manufacturing process on the relative percentages of leukocytes (apheresis material [APH], successive buoyant density gradients [BDS77 then BDS65], and final product [FP]) is demonstrated in the figure 4 below. Overall, the relative percentages of leukocytes in the product changes little from the starting material. Variability between lots remained relatively constant between the different manufacturing stages. The applicant has also shown in the BLA that the relative percentages of leukocytes in the product did not differ significantly between different clinical trials. Figure 4 also highlights the substantial variation in the relative percentages of leukocytes between lots, and that the manufacturing process does not alter this inherent variability.

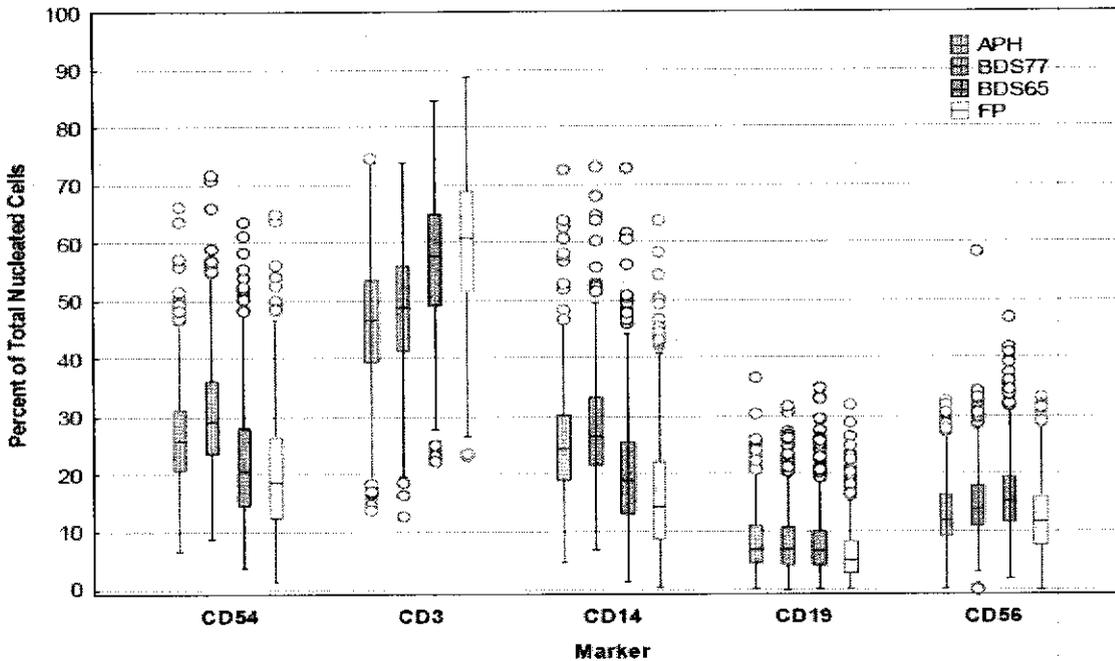


Figure 4. Percentage of leukocyte populations at various manufacturing stages of sipuleucel-T.

Numbers are calculated based on the total nucleated cell counts. Whereas all cell populations decreased when measured by total nucleated cell count, when assessed by percentage of total cell, CD3 positive cells increased. APH = leukapheresis, BDS77 = post first buoyant density centrifugation, BDS65 = post second buoyant density centrifugation just before incubation, FP = final product.

The consequences of CD3 positive, CD19 positive and CD56 positive cells remaining in the final product are unclear. Based on the observed variability in the relative percentages of leukocytes, establishing exclusion limits for the numbers of non-CD14 positive or non-CD54 positive cells could have a significant impact on the number of lots that would meet the current lot release criteria.

Cell Number

As stated above, the manufacturing process does not significantly alter the composition of this mixed population of leukocytes. In characterizing cell therapy products it is important to not only consider cell composition, but cell number, as that impacts the dose of the product.

The number of cells that are obtained from the leukapheresis varies widely. The applicant has established an in-process criterion for the minimum number of total nucleated cells (TNC) in the apheresis product that must be obtained in order to initiate the manufacture of sipuleucel-T. However, no upper limit is established for TNC count. A decrease in the numbers of T, B, NK, and monocyte populations is observed after each of the major manufacturing steps (Figure 5). However, the relative percentages of the populations do not change significantly at any step. In addition, there is no significant *in vitro* proliferation of the cells.

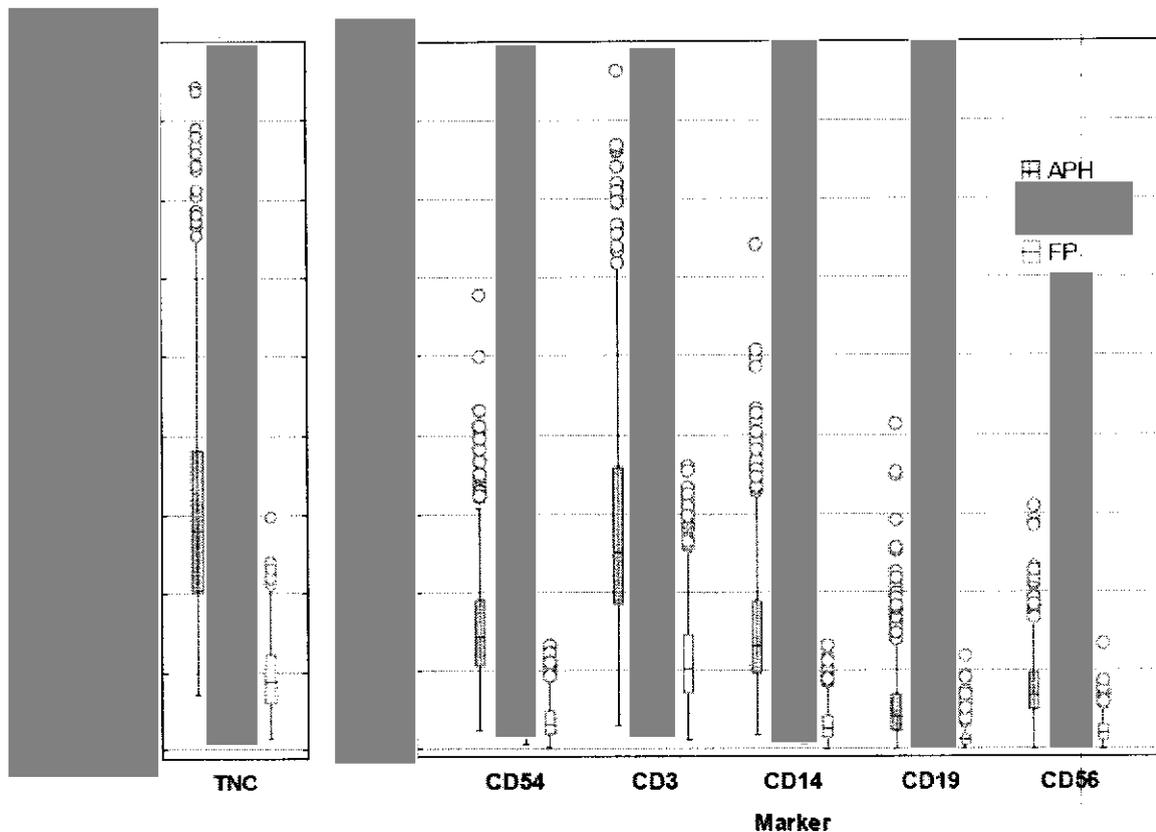


Figure 5. Leukocyte total cell numbers present within the starting leukapheresis material, processed cells, and the final product for autologous vaccination into patients.

These plots show the total cell counts for the several cell types at various manufacturing stages. APH = leukapheresis,

FP = final product. Data is based on 526 lots.

The range in TNC number in lots of sipuleucel-T is very broad and is largely attributable to the variability in the incoming apheresis material, not the process.

the relative percentage of each does not change appreciably in the final product. This is also reflected in Fig 4. The broad range in cell number will impact the total number of cells administered per dose. The applicant has not established criteria for minimal or maximal total nucleated cell dose in the final product. The only requirement for TNC is the minimum limit established for the apheresis material. A dose of sipuleucel-T is based on a minimum number of CD54 positive cells. An analysis of 1135 lots shows a greater than 180 fold difference between the highest and lowest numbers of CD54 positive cells in the final product.

In summary, the data on product cellular composition and cell number demonstrates very large inherent product variability. It also demonstrates that while lot to lot variations of the vaccine could be substantial, even from different vaccination lots from the same patient, that overall the manufacturing process is consistent. The applicant no longer evaluates the percentage of CD3, CD19, CD14, and CD56 positive cells during processing or in the final product.

PRODUCT POTENCY

While the manufacturing process is not designed to change the relative percentages of leukocyte populations in the final product, it is designed to activate the antigen presenting cells. The proposed mechanism of action is that antigen presenting cells will stimulate an *in vivo* cellular immune response against the tumor. The antigen presenting cells are not expected to directly kill tumor cells, but instead are expected to activate antigen-specific T cells. T cell recognition requires that the antigen presenting cells take up antigen, process it, and present it as peptides bound to major histocompatibility complex (MHC) molecules on the cell surface. T cell activation requires that the antigen presenting cells also provide a secondary stimulation to the T cells, which is only done by activated antigen presenting cells. This "second signal" comes through costimulatory molecules expressed on the surface of antigen presenting cells. As described below, costimulatory molecules are defined broadly and may include CD54, CD40, and CD86.

The ideal potency assay for a product with this proposed mechanism of action would be to measure the ability of the antigen presenting cells to induce antigen-specific T cells *in vitro*. This is not feasible for sipuleucel-T. Sipuleucel-T has an [REDACTED] shelf life and a typical assay to generate antigen specific T cells *in vitro* would take a minimum of several days. It also would not be feasible to generically measure antigen presentation due to HLA restriction in presentation of antigen. Each patient would have a different HLA type (i.e. would use different MHC molecules) and may differentially use the various peptide fragments. Instead of a T cell based potency assay, the applicant developed an assay that uses flow cytometry to measure CD54 expression as a lot release potency assay. This assay can be performed for lot release and is also reproducible and quantitative. The potency assay includes a measure of total CD54 positive cells and the increase in CD54 expression during *in vitro* culture as measured by mean fluorescence intensity (referred to as CD54 upregulation). The rationale for this assay includes:

- CD54 is present on antigen presenting cells in sipuleucel-T
- CD54 upregulation indicates a biological activity of sipuleucel-T
- CD54 positive cells are responsible for antigen uptake and presentation in sipuleucel-T

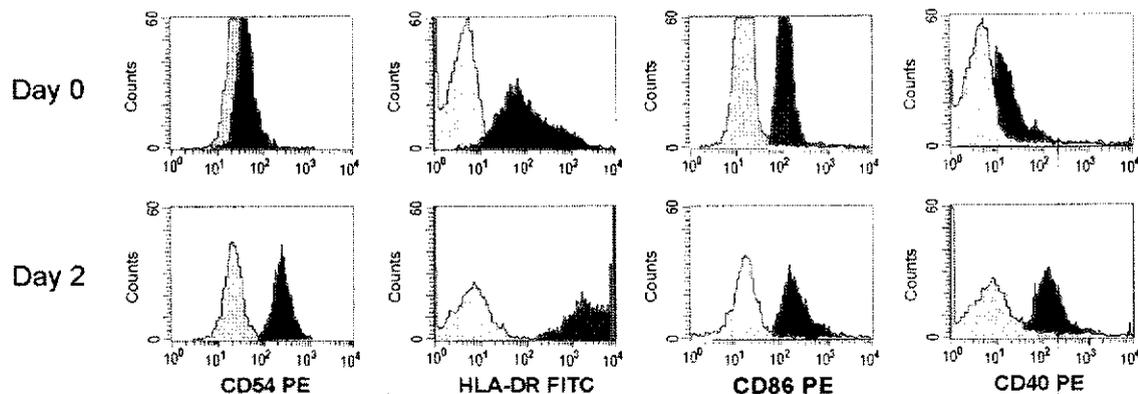
Some of the data provided by the applicant to support the choice of this assay are described below. While this potency assay will serve as relative measure of the biological activity of the product, it is not designed to predict an immune response or patient outcome.

CD54 upregulation during *in vitro* culture

As mentioned above, the apheresis is subjected to two rounds of buoyant density centrifugation to remove red blood cells, platelets, and granulocytes. The removal of granulocytes will help enrich the final product for cells involved in antigen-specific responses. Since neutrophils and eosinophils (both of which are granulocytes) express the GM-CSF receptor (Guthridge et al., 1998) and have phagocytic function, it is advantageous to remove them so that the added PA2024 protein can be targeted more directly to antigen presenting cells. Targeting the PA2024 protein to the antigen presenting cells helps to accomplish the two purposes of the protein: first, to activate the antigen presenting cells. Second, to provide antigen that will be presented to the T cells.

In order to demonstrate the ability of PA2024 to activate the antigen presenting cells, the

expression of several costimulatory markers by flow cytometry on cells before and after culture was measured. The data in the figure below (Figure 6) shows that several costimulatory molecules were expressed at a higher level after *in vitro* culture with PA2024 (Small et al., 2000).



Small, et. al. 2000

Figure 6. Upregulation of cell surface markers after culture with PA2024.

Evaluation by flow cytometry of APC activation by PA2024. The upregulation of cell surface molecules before and after culture of leukapheresed cells with PA2024 was measured by flow cytometry. Shown are flow cytometry histograms indicating the level of expression of either CD54 (also known as ICAM-1 and expressed on APC rich leukocytes), HLA-DR (MHC class II), CD86 (an APC costimulatory marker expressed on monocytes and dendritic cells), and CD40 (monocytes and B cells). Background antibody staining is shown in gray and specific staining is indicated in red. Results are from a phase I/II trial performed by the applicant (Small, et al. J. Clin Oncol. 2000 Dec 1;18(23):3894-903).

CD54 was chosen as a representative marker to measure the activation of antigen presenting cells. CD54, also known as ICAM-1 is a cell adhesion molecule that binds to LFA-1 present on T cells and other cell types (Springer, 1990). CD54 is highly expressed on monocytes and it is also present on many other peripheral blood cell populations including those in sipuleucel-T. The expression of CD54 by the various leukocyte populations in the final product can be seen in figure 8 below. The measure of activation is based on the ratio of the relative CD54 expression as measured by mean fluorescence intensity after culture compared to the CD54 expression before culture with PA2024.

In order to demonstrate whether the affects of PA2024 were attributable to the GM-CSF portion of the protein, the applicant measured the upregulation of CD54 with media alone, GM-CSF in media, or PA2024 in media. As shown in figure 7 below, the GM-CSF alone stimulates CD54 upregulation to the same extent as PA2024 indicating that the GM-CSF portion of the protein is most likely responsible for the activation of the antigen presenting cells. Additional data is also included in the BLA (but not shown here) to demonstrate that both cytokine production and the ability to stimulate an allo-mixed lymphocyte reaction response by the APC can be stimulated by either PA2024 or by GM-CSF alone. The additional data also support the hypothesis that the activation of APC by PA2024 is functionally important. In contrast, data shown below in figure 8 demonstrates that antigen presenting cells that receive GM-CSF alone do not stimulate antigen-specific T cells showing the critical role of providing the cancer antigen with the GM-CSF.



Since the proposed mechanism of action involves APC, it was important to demonstrate that CD54 upregulation was a measure of APC activation and not a general affect. The upper panel of figure 8 below, shows the cells that are detected as CD54 positive cells. This panel shows that the majority of CD54 positive cells are monocytes (CD14 positive) and not T, B, or NK cells (CD3, CD19, and CD56, respectively). In addition, the upper panel of figure 8 shows that *in vitro* culture does not change the relative distribution of CD54 positivity among the cell types. The lower panel in figure 8 shows the expression of a number of costimulatory molecules that are also present on CD54 positive cells before and after culture. CD54 positive cells before *in vitro* culture with PA2024 are also HLA-DR positive, while they are predominantly CD40, CD80, and CD86 negative. After culture, a portion of CD54 positive cells become positive for these other molecules.

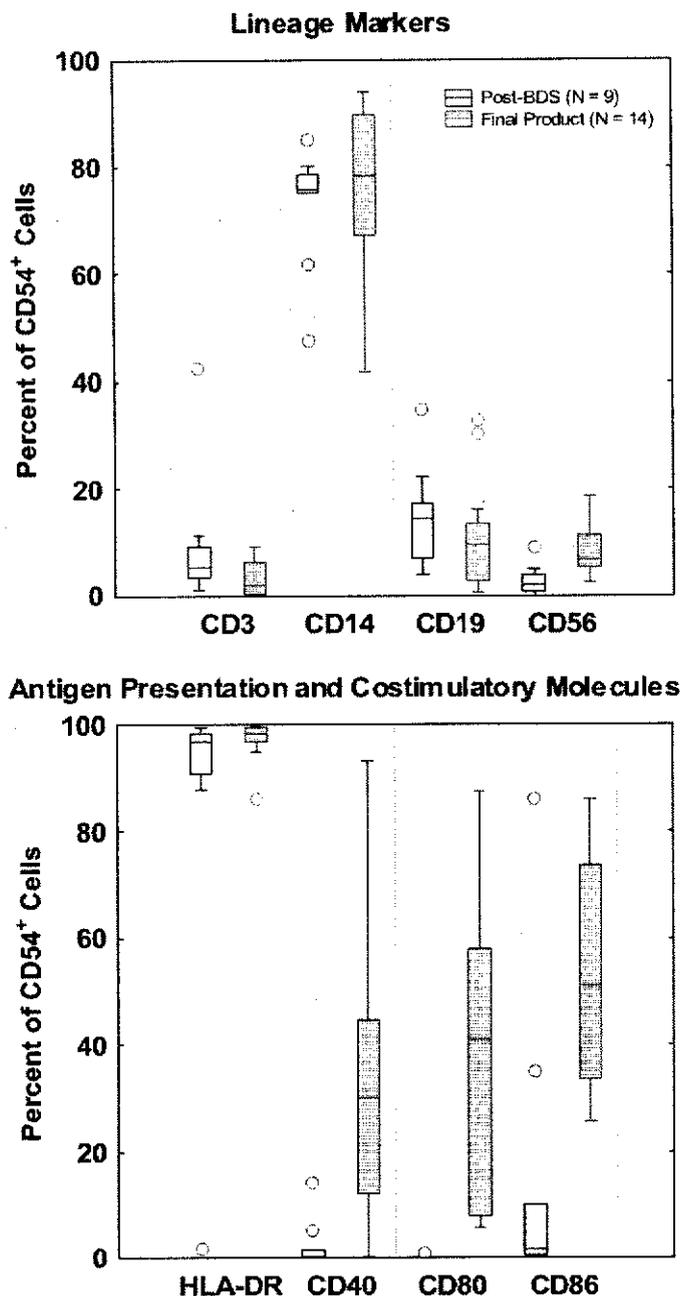


Figure 8. Expression of cell surface markers in the CD54 positive cell population before and after *ex-vivo* culture with PA2024.

As indicated in the lineage panel, CD54 positive cells as defined by the applicant, represent predominantly monocytes. The median percent of CD54 positive and CD14 positive cells is approximately the same before and after stimulation with PA2024, though the variability increases. The level of expression of MHC class II HLA-DR and 3 co-stimulatory molecules (CD40, CD80, and CD86) on CD54 positive cells was also determined. A substantial increase in all 3 costimulatory molecules expression was seen following incubation, suggesting the APC had become activated. As expected, HLA-DR levels were also high. Open bars represent pre-culture cells, shaded bars represent the final product. Whereas CD86 did not increase substantially when evaluated among the total cell population, when the analysis was restricted to CD54 positive cells a significant, though highly variable, increase in CD86 expression was seen. HLA-DR expression which was already high increased only slightly. Expression of MHC class I was not evaluated.

This group of studies supports the idea that APC are activated during the *in vitro* culture with PA2024. The data demonstrate that activation is largely attributed to the GM-CSF portion of PA2024. CD54, along with other costimulatory markers, is upregulated during the *in vitro* culture. The CD54 positive cells as measured by the applicant are predominantly CD14 positive.

Antigen presentation by CD54 expressing cells

Since the final product represents a mixed population of cells and the cumulative APC fraction is not the major cell type present, it was important to demonstrate the potential contribution of different populations of leukocytes. Various fractions of cultured cells were evaluated to determine the relative contribution of each subpopulation to antigen uptake and to antigen-specific T cell stimulation.

In order to determine which populations of cells are responsible for taking up the antigen, sipuleucel-T was incubated with fluorochrome FITC labeled PA2024 and sorted by flow cytometry. The figures below show the uptake of the FITC labeled PA2024 by the various cell types present in the product. Although not depicted here, data has also been submitted that shows that uptake can be competitively inhibited with a control GM-CSF fusion protein.

Figure 9 panel A shows that CD54 positive cells will predominantly take up the FITC-labeled PA2024. Panel B shows that CD40 positive cells also take up the antigen. This indicates that cells which are activated by the PA2024 antigen are also involved in taking up the antigen. Figure 10 shows the various cell types in sipuleucel-T and the ability of each to take up antigen. The FITC-labeled PA2024 is predominantly taken up by monocytes (panel A), is taken up very weakly by T cells (panel B), and does not appear to be taken up by B cells and NK cells (panels C and D, respectively).

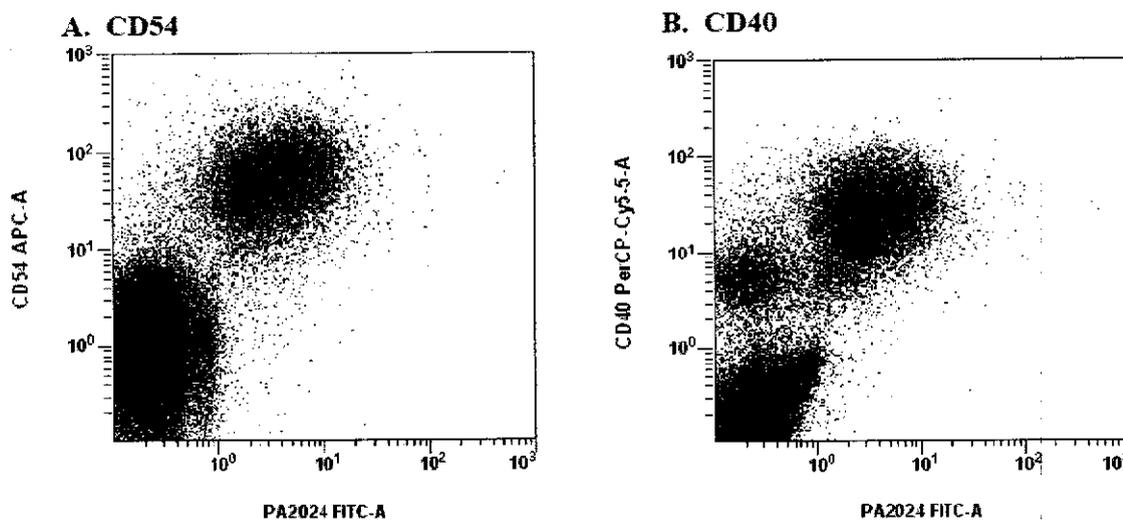


Figure 9. Correlation of Immune Activation-Associated Markers with PA2024-FITC Uptake.

Cells were isolated from a healthy donor APH at full scale according to the standard manufacturing process. The resulting post-BDS cells were cultured at a [REDACTED] PA2024 (containing 1% to 5% PA2024-FITC) at 37°C with 5% CO₂. [REDACTED]

[REDACTED] After culture, the cells were harvested by centrifugation and stained with fluorescently-labeled antibodies specific for cell surface markers. Multicolor flow cytometry was then used to determine which of these cell surface molecules were expressed on the cells that had taken up FITC-labeled PA2024. To examine specific staining of live cells, the dead cells were excluded from analysis by gating out the low forward-scatter population. As shown in Panel A, PA2024 uptake correlates closely with CD54 expression. All of CD54 positive cells incorporate PA2024, and all the cells that incorporate PA2024 are CD54 positive. Similarly, the cells that incorporate PA2024 are also CD40 positive (Panels B) and HLA-DR positive (data not shown).

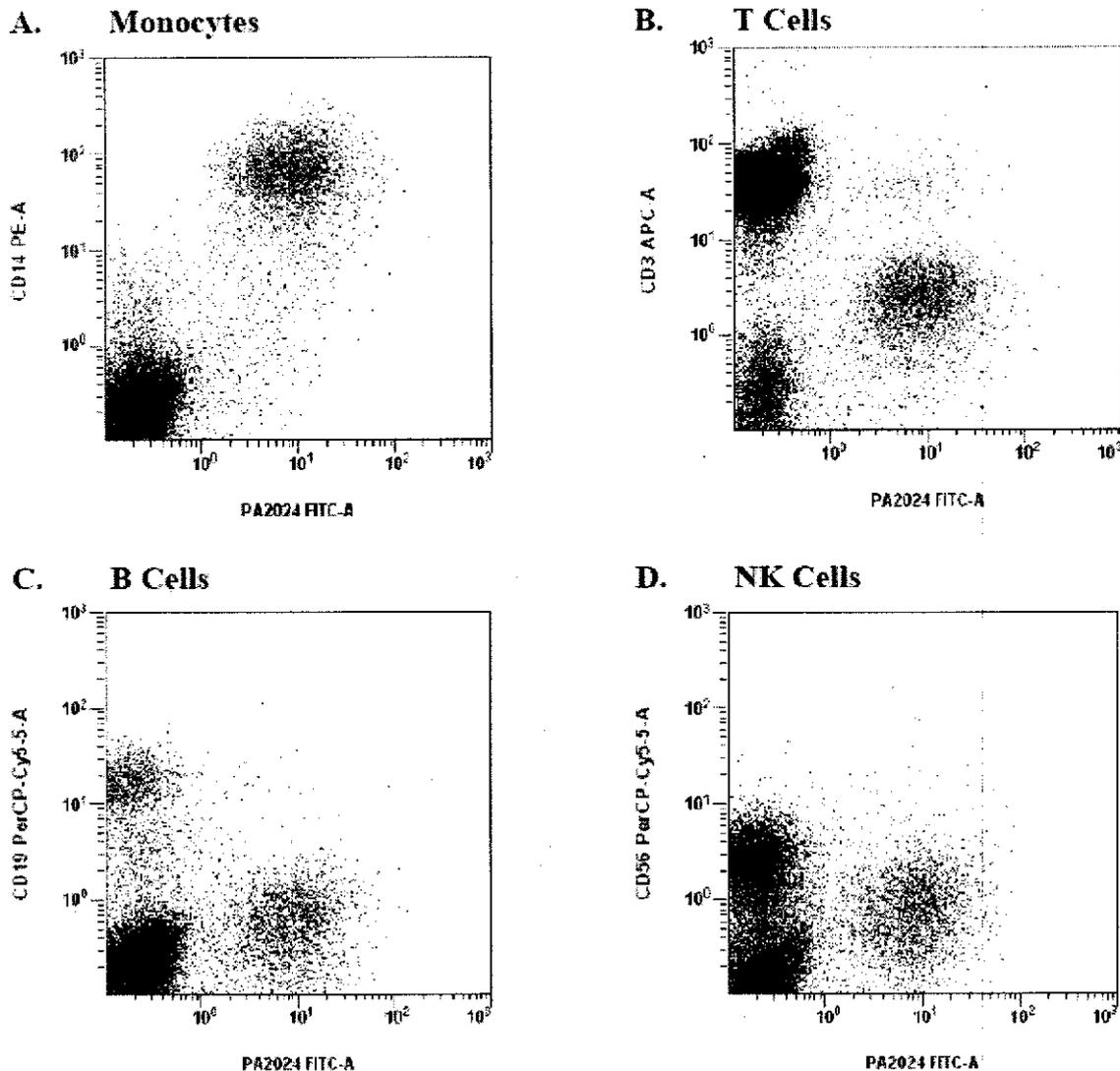


Figure 10. Correlation of Lineage Markers with PA2024-FITC Uptake.

These experiments were conducted as described in the previous figure. The results in Panel A show that monocyte-derived CD14 positive cells take up PA2024 antigen. In addition, a small number of cells aside from CD14 positive APCs take up PA2024. Panels B, C, and D show that most T cells (CD3 positive cells), B cells (CD19 positive cells) and NK cells (CD56 positive cells) do not take up PA2024 antigen. These results are representative of multiple experiments performed with cells from more than 10 healthy donors.

The applicant also submitted data to support the role of the CD54 expressing cells in antigen presentation. To accomplish this, the company created CD4 positive T cell hybridomas that are specific for HLA-DR1 restricted peptides from the PAP protein. These hybridomas produce IL-2 when stimulated by antigen presenting cells presenting PAP in the context of HLA-DR1. To perform the assay, T-hybridoma cells are co-cultured with APCs, and then the culture supernatants are assayed for IL-2. For these assays, HLA-DR1 positive donors are used to generate lots of sipuleucel-T. Cells from these lots are sorted into subpopulations and then tested for their ability to induce IL-2 production by the hybridoma. While this assay is able to measure antigen processing and presentation, it does not necessarily tell us about the costimulatory potential of the APC. It also will not inform us about the ability of other HLA haplotypes to present PAP epitopes.

Shown below are representative data from two donor lots of cells. The data shows that CD54 expressing cells (figure 11) are able to induce IL-2 production by the hybridoma indicating that those cells have the ability to take up, process, and present the antigen to T cells. In contrast, CD2 (T cells and NK cells), CD19 (B cells) and CD56 (NK cells) did not stimulate IL-2 production by the hybridoma (figure 12).

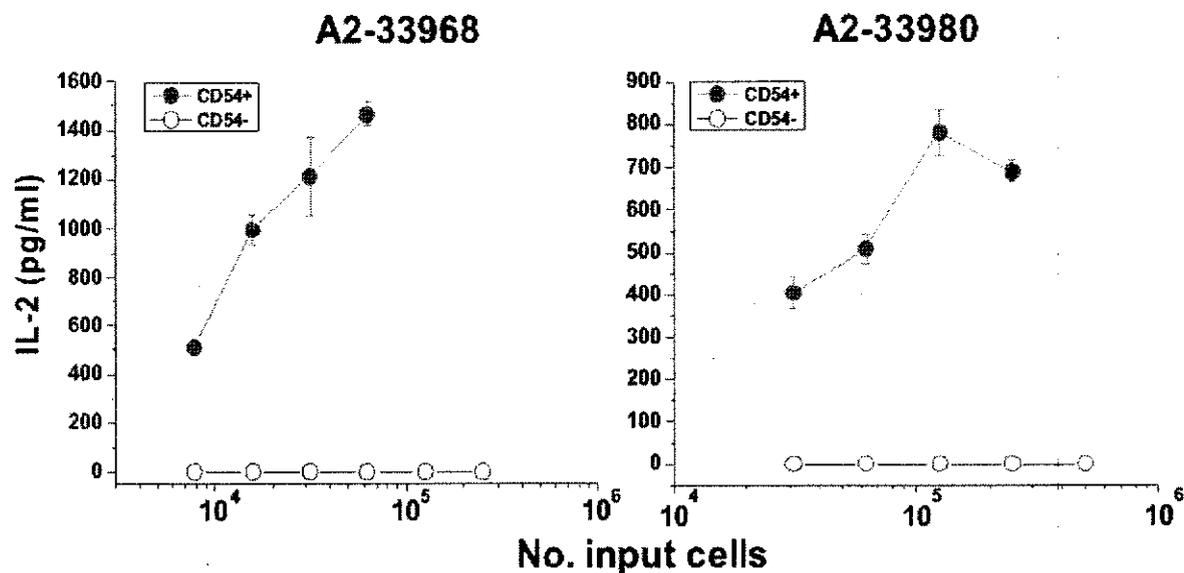


Figure 11. Antigen presentation to PAP-specific T cell hybridomas resides in the CD54 cell population.

The CD54 positive (CD54+) and CD54 negative (CD54-) fractions of harvested cells were assayed for PAP antigen presentation. These assays were performed multiple times, using cells from healthy, HLA-DR1+ donors. In this figure, the number of stimulator cells (CD54- or CD54+) is shown on the X-axes. The hybridoma response, measured in terms of IL-2 secretion, is shown on the Y-axes. The error bars show the standard deviation of triplicate determinations. These results show that the CD54 positive cell population contains all of the PAP antigen presentation activity in sipuleucel-T.

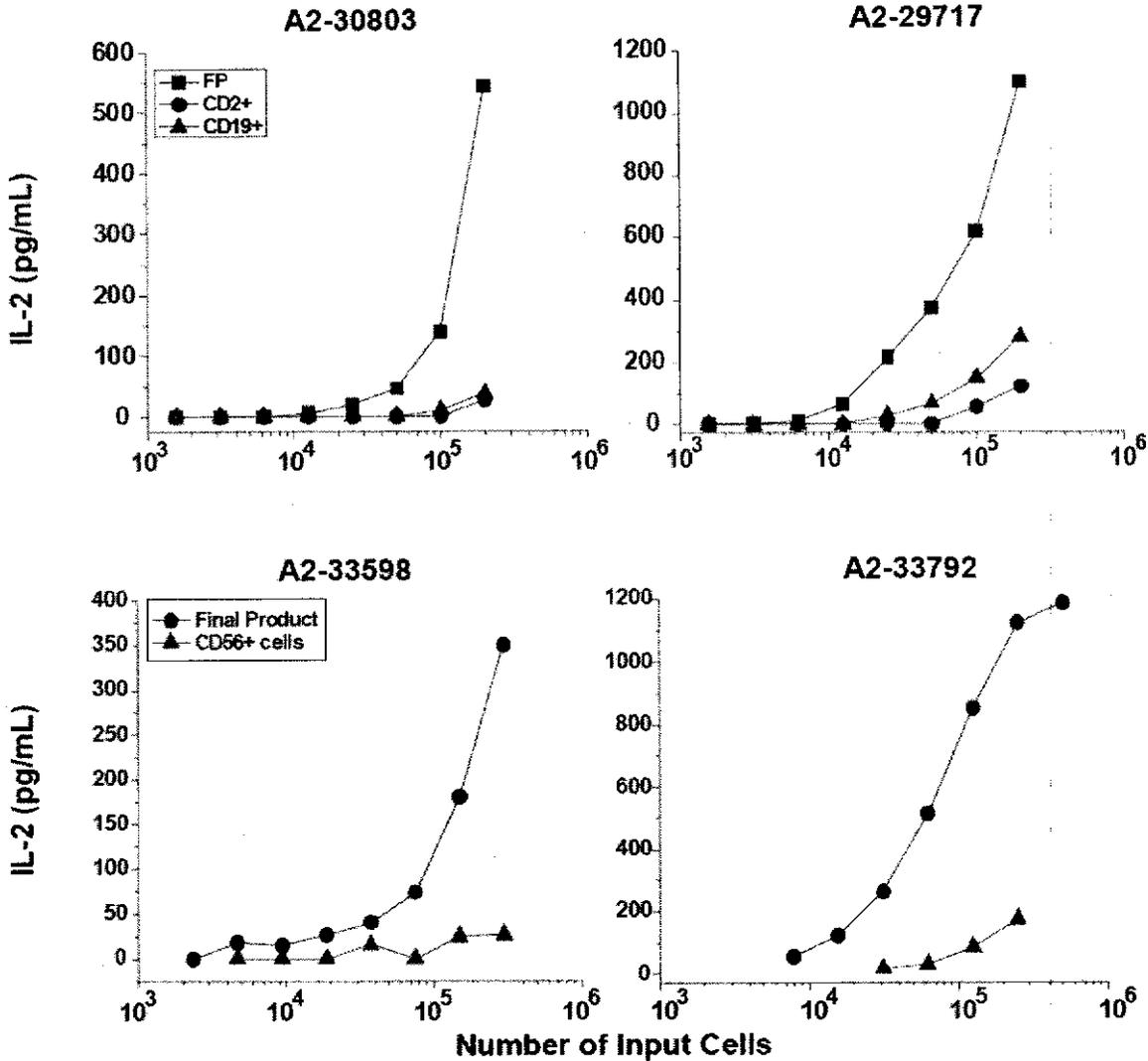


Figure 12. PAP antigen presentation by other cell types.

Sorting experiments were performed to determine if B cells, T cells and NK cells present antigen in the PAP-specific antigen presentation assay. Because staining for CD3 expression could activate T cells via CD3 cross-linking, the cells were stained for CD2 instead. It is important to note that CD2 is expressed on both T cells and NK cells. Sipuleucel-T was prepared from 4 healthy donors APH according to the standard manufacturing process. Final product cells were stained with antibodies to CD2 (T cells and NK cells) or CD19 (B cells). Stained cells were isolated by FACS, and the sorted subpopulations were compared with unsorted final product in triplicate using the PAP-specific antigen presentation assay. Hybridoma responses were measured using the IL-2 ELISA.

To demonstrate the specificity of the response to PAP, the hybridoma assay was performed using antigen presenting cells that were cultured with a GM-CSF fusion protein that does not contain prostate antigen (BA7072) or with PA2024. Figure 13 below shows that PA2024 is required to stimulate the PAP-specific hybridoma cell line.

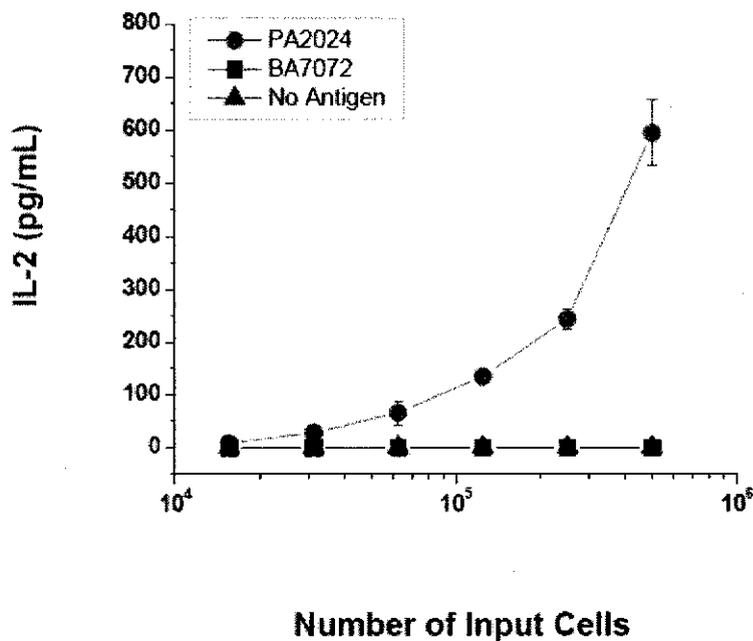


Figure 13. Effect of GM-CSF on PAP Antigen Presentation.

Post-BDS cells were prepared from a healthy donor APH and cultured in 1) AIM-V® medium alone (No antigen), 2) AIM-V® medium with E. coli-derived human GM-CSF (R&D Systems) (BA70720, and 3) AIM-V® medium with PA2024 (PA2024). After culture, cells were put into the hybridoma assay to determine their ability to stimulate an antigen specific T cell response. Culture with PA2024 was required for PAP-specific antigen presentation activity. Culture with BA7072 or AIM-V® medium alone does not yield any PAP-specific antigen presentation activity.

This antigen uptake and presentation data shows that the CD54 positive cells are the primary cell type responsible for both of these functions. The other cell types present in the final product do not contribute significantly to either antigen uptake or presentation. Therefore, while CD54 expression on the final product is not a measure of antigen presentation, these development data support the presence of CD54 cells as an indication that there are cells with antigen uptake and presentation ability in the product.

CD54 upregulation and CD54 cell numbers as a measure of potency

As a measure of potency for the product, the applicant is measuring both the upregulation of CD54 expression during *in vitro* culture (comparison of level of expression prior to and after culture with PA2024) along with total CD54 positive cell counts. As a flow cytometry method, CD54 expression can be measured in a relatively rapid and reliable fashion. This is unlike the antigen presenting assays described above, which are lengthy bioassays that could not be performed for release of the product due to the short shelf life.

The applicant also submitted data from their clinical manufacturing experience that demonstrates the results of product potency measures during clinical trials. The data in figure 14 describe the level of upregulation seen for clinical lots (panel A) as well as the total number of CD54 positive

cells (panel B). This figure provides data from each of the 3 doses of the treatment cycle. It is important to note that while the number of CD54 positive cells present in the final product did not increase with subsequent vaccinations, the median level of CD54 upregulation did increase between the first vaccination and the week 2 vaccination. This indicates that the response to PA2024, at least *in vitro*, is increasing with subsequent doses of product. These data describe the applicant's experience measuring the potency of sipuleucel-T.

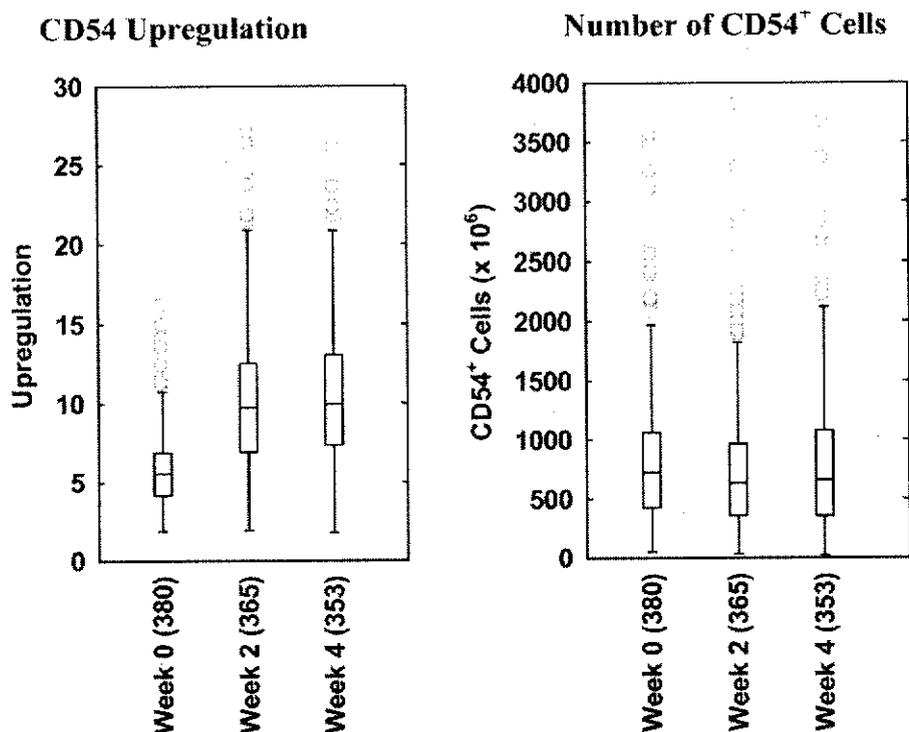


Figure 14. CD54 cell number and CD54 upregulation present in the final product.

Changes in the number of CD54 positive cells in the final product, and fold upregulation of the level of expression CD54 on the final product relative to cells prior to culture with PA2024) was compared between lots generated for different vaccination phases. The number in parenthesis is the number of lots analyzed.

These data describe the ability of the manufacturing process to upregulate the expression of CD54 on a subset of patient leukocytes. The upregulation of CD54 serves as a surrogate measure for the biological activity of the APC. CD54 upregulation is representative of the cells' ability to respond to GM-CSF and become activated. The data also indicates that CD54 positive cells in sipuleucel-T are responsible for antigen presentation. Together, these data form the basis for the applicant's justification for CD54 as the measure of product potency.

REFERENCES

Cunha,A.C., Weigle,B., Kiessling,A., Bachmann,M., and Rieber,E.P. (2006). Tissue-specificity of prostate specific antigens: Comparative analysis of transcript levels in prostate and non-prostatic tissues. *Cancer Letters* 236, 229-238.

*Guthridge,M.A., Stomski,F.C., Thomas,D., Woodcock,J.M., Bagley,C.J., Berndt,M.C., and Lopez,A.F. (1998). Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors. *Stem Cells* 16, 301-313.

Nguyen,X.D., Eichler,H., Sucker,A., Hofmann,U., Schadendorf,D., and Kluter,H. (2002). Collection of autologous monocytes for dendritic cell vaccination therapy in metastatic melanoma patients. *Transfusion* 42, 428-432.

Rice,A.M., Jones,K.L., and Hart,D.N. (2004). DC preparations for therapy. *Cytotherapy*. 6, 99-104.

Shaz,B., Goodarzi,K., Malynn,E., and Uhl,L. (2006). Improved strategy for mononuclear cell collection for donor lymphocyte infusions. *Transfusion* 46, 1044-1048.

Small,E.J., Fratesi,P., Reese,D.M., Strang,G., Laus,R., Peshwa,M.V., and Valone,F.H. (2000). Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. *J. Clin. Oncol.* 18, 3894-3903.

*Springer,T.A. (1990). Adhesion receptors of the immune system. *Nature* 346, 425-434.

Wolf,C.E., Meyer,M., and Riggert,J. (2005). Leukapheresis for the extraction of monocytes and various lymphocyte subpopulations from peripheral blood: product quality and prediction of the yield using different harvest procedures. *Vox Sang.* 88, 249-255.

*Full-text versions of these documents are included with the briefing document