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ORIGINAL ARTICLE

CD54 is a surrogate marker of antigen presenting cell activation

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Abstract There is no single universally accepted hallmark of antigen presenting cell (APC) activation. Instead a variety of methods are used to identify APCs and assess their activation state. These activation measures include phenotypic methods [e.g., assessing the increased expression of surface markers such as major histocompatability (MHC) class II] and functional assays (e.g., evaluating the enhanced ability to take up and process antigen, or stimulate naïve T cells). Sipuleucel-T is an investigational autologous active cellular immunotherapy product designed to stimulate a T cell immune response against human prostatic acid phosphatase (PAP), an antigen highly expressed in prostate tissue. Sipuleucel-T consists of peripheral blood mononuclear cells (PBMCs), including activated APCs displaying epitopes of PAP. In order to develop a robust reproducible potency assay that is not hampered by MHC restriction we have developed a method to simply assess the biological activation of antigen presenting cells (APCs). In the course of sipuleucel-T characterization, we analyzed various phenotypic and functional parameters to define the activation state of APCs obtained from peripheral blood. Flow cytometric assays revealed that CD54+ cells are responsible for antigen uptake, and that expression of CD54 predominantly localizes to APCs. Costimulation, as measured by an allogeneic mixed lymphocytic reaction (allo-MLR) assay, showed that activity was restricted to the CD54+ cell population. Similarly, CD54+ cells harbor all of the PAP-specific antigen presentation activity, as assayed using a PAP-specific HLA-DRβ1-restricted T cell hybridoma. Finally we show that CD54 expression is substantially and consistently upregulated on APCs during culture with a GM-CSF fusion protein, and that this upregulation activity can be quantified. Thus these data support the use of CD54 upregulation as a surrogate for assessing human APC activation and validates its utility as a potency measure of sipuleucel-T.

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Introduction

Cellular immunotherapies represent an emerging and promising approach to treat cancer. However, these therapies face many challenges, not the least being the assessment of potency, a product release measure that is required by the Food and Drug Administration (FDA) [19]. Potency has been defined by The International Conference on Harmonization (ICH) as “the measure of biological activity, using a suitably quantitative biological assay, based on the attribute of the product that is linked to the relevant biological properties” (ICH Guideline Q6B, 1999). Potency is therefore a quantitative assessment of the product’s biological activity as measured via some presumed mechanism of action.

In the case of cell-based therapies, a test of the expected mechanism of action of the therapeutic product could appropriately serve as the basis for potency [13]. In general, for antigen presenting cell (APC) based autologous cellular immunotherapies, cells from the peripheral blood may be cultured with a target antigen before re-infusion, with the precept that a target-specific immune response will be stimulated in the patient’s body to yield a T cell mediated therapeutic effect. As the initial events for generating an antigen-

specific immune response are dependant upon APC activity, the potency measure would most easily be focused on APCs.

A two-signal hypothesis is commonly used to describe the activation of T cells by APCs. The first signal is provided by epitopes generated from processed antigen that are presented, in the context of surface major histocompatibility complex (MHC) molecules on the APCs, to the antigen receptor complex on T cells. The second signal is provided by costimulatory molecules on the APCs that bind to ligands on the surface of T cells. While both signals provide direct evidence of APC functionality, there are obstacles to detecting the display of processed antigen. For example, detection of antigenic epitopes complexed with an HLA molecule requires a library of antigen-specific T cells with varied HLA restrictions. This strategy is impractical in that it necessitates generation and validation of multiple assays to accommodate all patients' HLA haplotypes [14].

Costimulatory activity has been classically assessed using the allogeneic mixed lymphocyte reaction (Allo-MLR) assay, which has served as the established standard for evaluating the functional ability of APCs [27]. However, as with many biological systems based around human cells there are numerous difficulties with this approach; for example, the stimulating alloantigens can vary greatly between batches of responding T cells and stimulating APCs. This intrinsic variability can cloud interpretation of data. Therefore an ideal strategy would be to use a surrogate marker of cellular activation that is correlative with the functional ability of the APC.

In the course of our studies we studied the expression of a number of costimulatory and activation markers that have been reported to be increased upon cellular activation [5, 9] and observed that CD54 expression was consistently increased upon APC after culture with fusion protein.

CD54, or ICAM-1, is a ligand for the leukocyte integrin complex CD11a/CD18 (LFA-1) and is expressed at differing levels on a variety of cells, including APCs, T cells and B cells [21, 31]. The adhesive interaction between CD54 and LFA-1 facilitates cell–cell contact [6] and is thought to strengthen the immune synapse [4]. In addition, experimental evidence also suggests that CD54 can be considered a costimulatory receptor [16]. The costimulatory effect of CD54 appears to depend upon cell identity; during MHC class I restricted antigen presentation, CD54 acts as a signaling molecule when expressed on APCs; however, on CD8+ T cells it behaves as a costimulatory receptor [7].

We show that quantitation of CD54 expression is an appropriate assay for potency in the autologous cellular immunotherapy product sipuleucel-T [25]. CD54 expression is thus a marker for APCs and therefore a surrogate marker of APC activation as measured by antigen uptake, processing and presentation as well as enhancement of costimulatory activity.

Materials and methods

Cell preparation and culture

Peripheral blood cells were collected by leukapheresis from healthy donors (Puget Sound Blood Center, Seattle, WA, USA) and were subjected to two buoyant density separations to isolate mononuclear cells as previously described [2, 3, 10, 24]. Cells were cultured in AIM-V medium (Invitrogen, Grand Island, NY, USA) for 36–44 h with either PA2024 [14], a recombinant fusion protein containing PAP and GM-CSF or with BA7072, a recombinant fusion protein comprised of Her2/neu sequences and GM-CSF [18]. After culture, the cells were harvested by centrifugation and then washed and resuspended in Lactated Ringer's solution, USP (LR—Hospira Inc, Lake Forest, IL, USA). In some experiments the PA2024 or BA7072 was spiked with 1% (w/w) fluorescein-isothiocyanate (FITC)-labeled PA2024 or BA7072, respectively. In accessory experiments cells were cultured with either TLR3, TLR4 or TLR7 ligands (all purchased from Invivogen, San Diego, CA, USA).

Cell sorting

Sterile samples of final product cells were washed in Dulbeccos-phosphate buffered saline (D-PBS) and stained with anti-human CD54 Phycoerythrin (PE) (Becton Dickinson, San Jose, USA). Cells were washed in D-PBS and adjusted to a target cell concentration of 15–20 $\times$ 10^6/mL. A sterile two-way sort of PE positive and PE negative cells was then conducted on the stained cells. In the case of cells incubated with PA2024-FITC, cells were sorted on the basis of their FITC signal. Sorting was performed on a FACSARIA™ instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), which was optimized for sorting using a 70-µm nozzle and 90% purity mode. Sorted positive and negative cell fractions were collected in sterile tubes containing complete IMDM (cIMDM) media (Invitrogen). After sorting cells were centrifuged and washed in cIMDM, enumerated, evaluated for cell viability, and analyzed on the FACSARIA™ cell sorter to measure purity of the sorted cell populations as described further below.

PAP and Her2/neu presentation assays

PAP-specific antigen presentation was assessed by means of Papillon a PAP-specific T-cell hybridoma [31], and Her2/neu antigen presentation was evaluated by Herder a Her2/neu-specific T-cell hybridoma; both cell lines are HLA-DR1β1 restricted murine T cell hybridomas. After culture with antigen cells from a HLA-DRβ1 donor were serially diluted in triplicate against 1 $\times$ 10^5 Papillon cells or Herder cells in 96 well flat bottom tissue culture plates (BD

Falcon, San Jose, CA). The plates were incubated for 24 h at 37°C and the supernatant was harvested and stored at −20°C. IL-2 production by the T cell hybriomas was assessed by measuring the production of IL-2 by IL-2 ELISA (Becton Dickinson).

Allogeneic-mixed lymphocyte reaction

Prior to or after culture with antigen, cells were irradiated (30 Gy) using a JL Shepard, Model 143 laboratory irradiator (San Fernando, CA, USA) and added to the wells of a 96 well round bottom plate in quadruplicate at an initial cell concentration of 5×10^5/well. Cells were serially diluted twofold down the plate, then 5×10^4 human CD3+ T cells purified from multiple donors were added as responders to each well. The plates were incubated for 6 days at 37°C and were pulsed with 0.5 μCi [^{3}H] thymidine (Perkin Elmer, Waltham) for the final 18 h. Cells were harvested onto glass fiber mats (Perkin Elmer, MA, USA) using a Tomtech cell harvester (Tomtec, CT, USA), read on a Wallac Betaplate 1205 plate counter (Perkin Elmer), and the incorporation of [^{3}H]thymidine was expressed as counts per minute.

Multicolor staining of the CD54 cell population

Cells were blocked with 10% normal mouse serum (Jackson Laboratories, Bar Harbor, ME, USA) in Dulbecco's Phosphate Buffered Saline (D-PBS, Life Technologies, Grand Island, NY, USA) and then washed with D-PBS. Cells were stained in combination with anti-CD54 PE, anti-CD14 PerCPCy5.5, anti-HLA-DR APC-Cy7 or with anti-lineage (anti-CD3, anti-CD14, anti-CD19, anti-CD56) cocktail-FITC in combination with anti-CD54-PE, anti-HLA-DR APC, anti-CD11c APC and anti-CD123 PerCP Cy5.5 (all antibodies were purchased from Becton Dickinson). Cells were incubated for 15–30 min at 4°C in the dark, after which time they were further washed with D-PBS and then resuspended in 1% paraformaldehyde. Fixed samples were acquired on a FACSARIA™ flow analyzer where up to 500,000 forward versus side scatter gated events were collected.

Determination of surface marker expression upregulation

The increase in surface marker expression was determined by flow cytometry. Prior to and after culture cells with antigen were blocked with 10% normal mouse serum in D-PBS and then washed with D-PBS. The cells were then stained with anti-CD80 FITC, CD54 PE, CD86 PE-Cy5, CD40 APC, HLA-DR APC-Cy7 (Becton Dickinson, San Jose, San Jose, CA, USA). Cells stained with isotype-matched fluorescent labeled control antibodies were used to discriminate background staining.

Stained samples were collected on a BD FACSAria™ flow cytomer where 200,000 events were collected and data analyzed using CXP software (Beckman Coulter, Fullerton, CA, USA). Increases in surface expression of the aforementioned markers were expressed as a ratio of post-culture geometric mean fluorescence divided by the pre-culture geometric mean fluorescence of each individual marker after subtraction of the signal obtained from cells stained with isotype matched control antibodies at each stage.

Determination of CD54 molecule expression

The number of CD54 molecules expressed on APC before and after culture was determined by flow cytometry staining. Before and after culture cells were blocked with 10% NMS in D-PBS and then stained with anti-CD54 antibody (Becton Dickinson) conjugated with 1 molecule of PE per antibody molecule (hereafter referred to as CD54 PE 1:1). An isotype matched control antibody (Becton Dickinson) also conjugated with PE at a ratio of 1 molecule of PE per antibody molecule was used to determine background fluorescence. A PE standard curve was established by running PE QuantiBRITE™ beads (Becton Dickinson) along with the stained cells and APC gating was employed as per standard guidelines [16]. The geometric mean fluorescence intensity of the CD54+ APC population was compared to a Quantibrite PE standard curve and the average number of CD54 molecules was thus derived.

Results

CD54 is an appropriate marker for antigen uptake and presentation

Initial experiments were performed to determine which cell surface marker best defined cells that assimilated antigen in sipuleucel-T. The antigen used was PA2024, a fusion protein composed of PAP fused to GM–CSF. The PAP portion of the molecule provides the tumor-associated antigenic peptides, and GM–CSF functions as an activator of APCs [23, 32]. Cells were incubated with PA2024 or PA2024 spiked with 1% FITC-labeled PA2024 and cultured as described in materials and methods. After culture, cells were stained with antibodies specific for molecules associated with antigen presenting cell function, e.g., HLA class II or costimulatory molecules (CD40, CD54 and CD86). Figure 1 is representative of multiple experiments demonstrating that antigen uptake, indicated by FITC fluorescence, was restricted to CD54+ and HLA-DR+ cells. Of note is the observation that in a number of experiments, not all HLA-DR+ cells

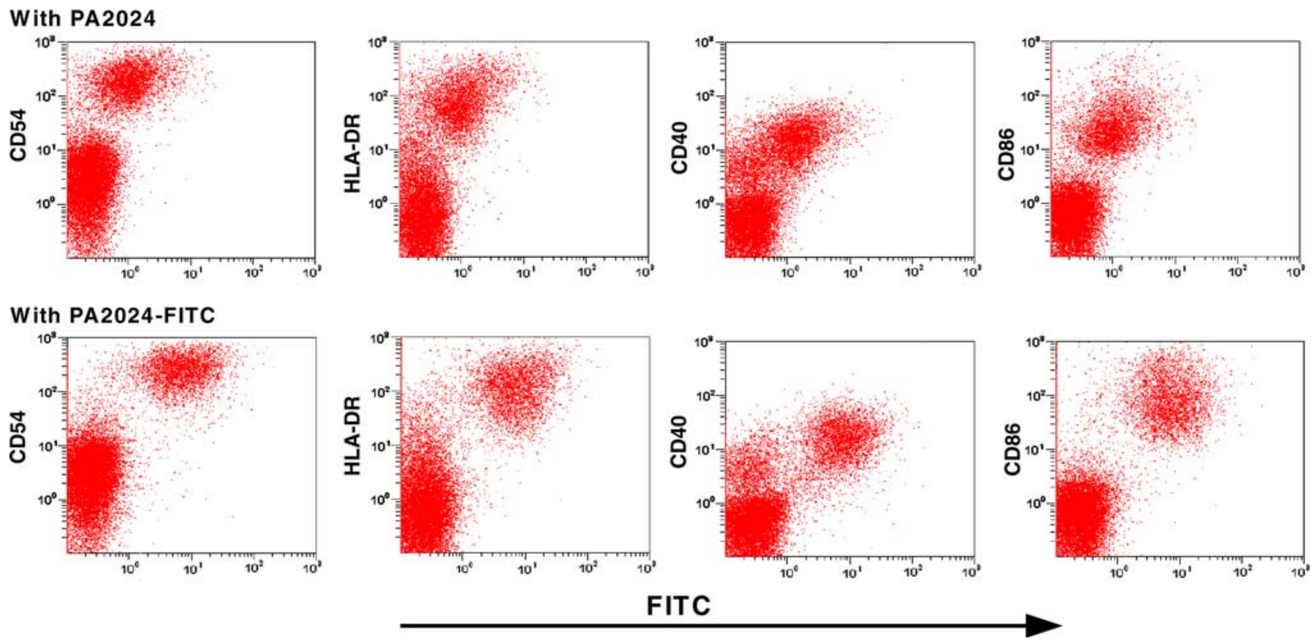


Fig. 1 FITC labeled PA2024 is assimilated preferentially by CD54+ cells. Cells were incubated with ether PA2024 or PA2024 spiked with 1% (w/w) FITC labeled PA2024 and after approximately 40 h cells were harvested as described in the materials and methods and surface stained for CD54, HLA-DR, CD40 and CD86. Stained cells were analyzed on a BD FACSAria™ flow cytometer and data is displayed using CXP software. The *top row* of flow plots represents cells cultured with unlabeled PA2024 and the *lower row* is from cells cultured with PA2024 spiked with FITC labeled PA2024. Data presented is from one representative set from multiple experiments

took up antigen as was also the case with CD40 expressing cells.

To determine whether the cells that took up antigen also processed and presented the antigen in an MHC restricted manner, the cell population that was positive for FITC-labeled PA2024 was isolated by cell sorting and used to stimulate PAP-specific T cell hybridomas. As shown in Fig. 2a, the APC function is contained entirely in cells that have taken up antigen, as defined by fluorescence. The same results were obtained with a HLA-DRβ1 restricted T cell hybridoma specific for a different PAP derived peptide (data not shown).

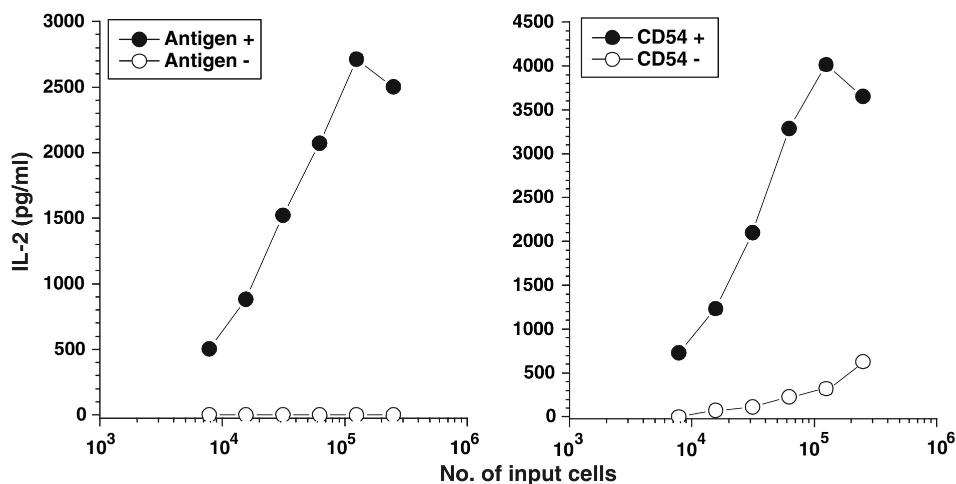


Fig. 2 CD54+ cells assimilate and present antigen. **a** Cells were incubated with PA2024 spiked with 1% (w/w) FITC labeled PA2024. After approximately 40 h cells were harvested and sorted on the basis of their FITC signal as described in Materials and methods. The two resultant cell populations (FITC+ and FITC−) were evaluated for expression of PAP epitopes using the PAP-specific Papillon hybridoma. FITC+ and FITC− cells were titrated in triplicate against 1×10^5 Papillon cells in 96 well flat bottomed plates. IL-2 production was measured by ELISA. **b** Cells cultured with PA2024 were sorted on the basis of CD54 expression. CD54+and CD54- cells were titrated in triplicate against 1×10^5 Papillon cells in 96 well flat bottomed plates and IL-2 production was measured by direct ELISA. Data presented is from one representative set from multiple experiments

Additional experiments to address whether the CD54+ cell population contained antigen presenting activity were performed. After culture with PA2024 cells were sorted into CD54+ and CD54- populations and the sorted cell populations were used to stimulate PAP-specific T cell hybridomas (Fig. 2b). In all sorting experiments PAP peptide presentation resided exclusively in the CD54+ cell population. Thus these experiments demonstrate that CD54 describes the cell population in sipuleucel-T that is responsible for antigen uptake and presentation, although this does not mean that that every CD54+ cell presents PAP epitopes.

CD54+ cells are phenotypically APCs

Having established that antigen uptake and presentation activity was restricted to the CD54+ cell population, we further characterized the phenotype of this cell population using multicolor flow cytometry. As APC are responsible for uptake and processing of soluble antigen and we had established that antigen-presenting activity was restricted to the CD54+ cell population, we focused on phenotyping the CD54+ population. Cells were stained with a combination of anti-CD54 and anti-HLA-DR, or anti-lineage cocktail antibodies in conjunction with anti-HLA-DR, anti-CD11c and anti-CD123 antibodies.

Figure 3 shows that the majority of the large CD54+ cells were of the monocyte lineage (CD14+); although plasmacytoid (lineage-, HLA-DR+, CD123+) and myeloid (lineage-, HLA-DR+, CD11c+) dendritic cells were also detectable in the large CD54+ cell population, albeit at very low frequencies. In addition, CD54+ cells were virtually all HLA-DR+ suggesting that they are classical APCs. The CD54+ cell population was also found to contain T cells (CD3+), B cells (CD19+) and NK cells (CD56+), although the relative percentage of these peripheral blood mononuclear cells was in the single digit percentage range (data not shown).

CD54+ cells have greater costimulatory activity after culture

We next evaluated whether the CD54 population had a functional impact on costimulatory activity. The PAP-spe-

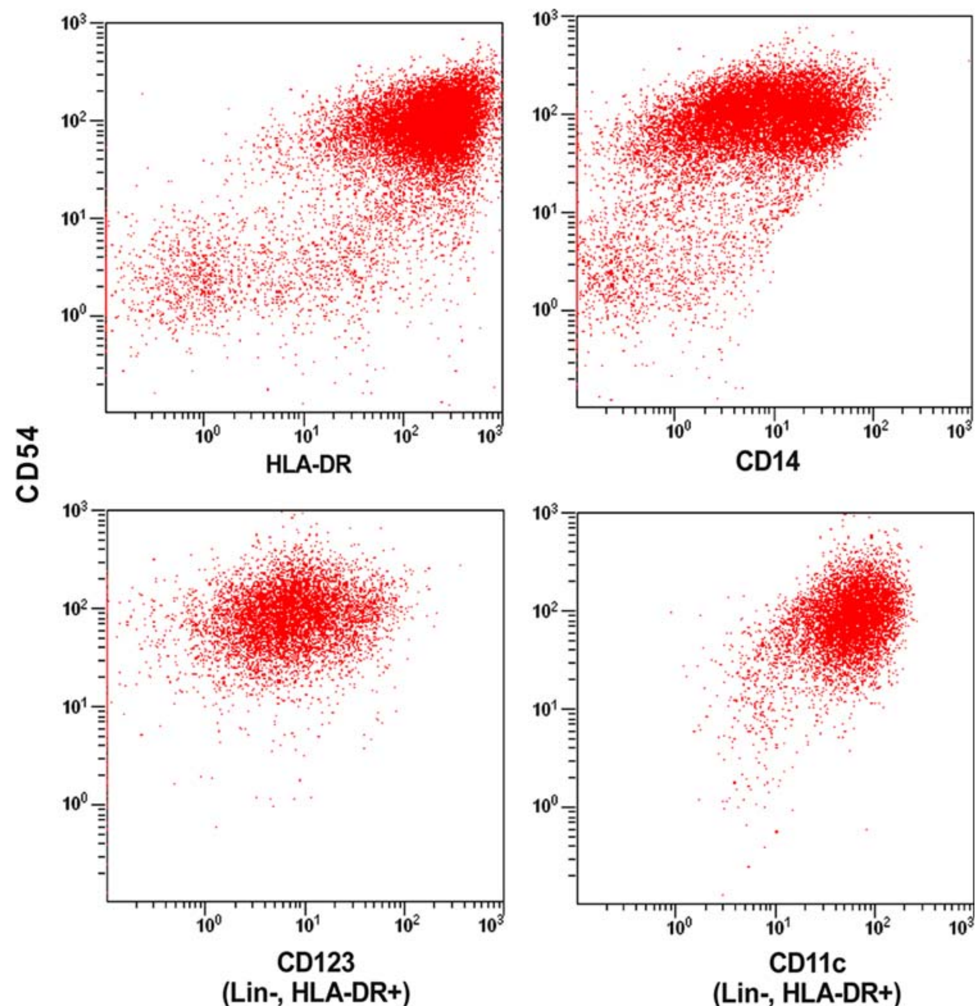


Fig. 3 Antigen presenting cells express CD54. Post culture cells were co-stained with anti-CD54 PE, anti-CD14 PerCPCy5.5, anti-HLA-DR APC-Cy7 or with anti-lineage cocktail-FITC in combination with anti-CD54-PE, anti-HLA-DR APC-Cy7, anti-CD11c APC and anti-CD123 PerCP Cy5.5. Stained cells were collected on a BD FACSAria™ flow cytometer where a total of 500,000 events were collected. Analysis was restricted to large cells (determined by forward scatter vs side scatter). In the case of cells stained for myeloid (CD11c) and plasmacytoid (CD123) DC markers analysis was based upon large cells that were lineage negative and HLA-DR positive. Data presented is a representative set from multiple experiments

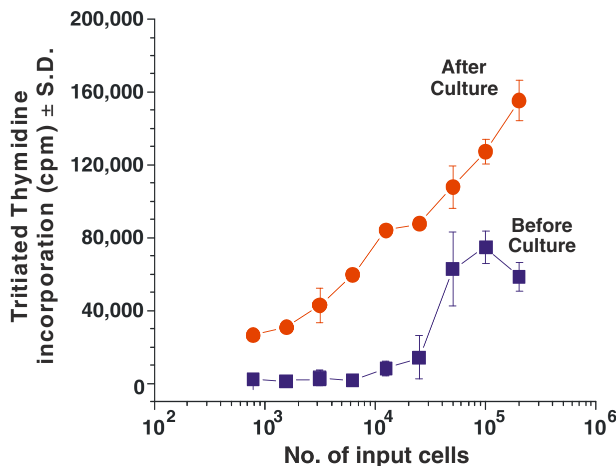


Fig. 4 CD54+ cells possess costimulatory activity, which increases after culture with the fusion protein PA2024. Prior to and immediately after culture with antigen, cells were sterile sorted on a BD FACSAria on the basis of CD54 expression. CD54+ sorted cells were irradiated and titrated in 96 well flat bottom plates in quadruplicate against 5×10^4 human CD3+ cells purified from multiple healthy donors. Plates were incubated for 6 days at 37°C with a pulse of 0.5 mCi [^{3}H] thymidine for the final 18 h. Cells were harvested and read on a betaplate counter. CD54- sorted cells did not have any stimulatory capacity as evidenced by the fact that incorporation of tritiated thymidine was below background levels (not presented). The data displayed is from one of two independent representative experiments

cific T cell hybridomas, similar to reports of other T cell hybridomas in the literature, have been demonstrated to be relatively insensitive to costimulatory molecule expression. Therefore alternative systems such as AlloMLR, have been utilized to evaluate the functional activity of APCs [22]. We evaluated the ability of CD54+ sorted cells to stimulate naïve T cells in the AlloMLR prior to and immediately after culture with PA2024. Similar to the hybridoma assay (Fig. 2), CD54- cells were devoid of stimulatory activity before and after culture (data not shown).

As shown in Fig. 4, costimulatory activity was greatly increased in the CD54+ cell population after culture with PA2024. Furthermore CD54 expression increased nearly tenfold during culture: before culture the CD54+ cell population expressed an average of 9,050 CD54 molecules per cell and after culture the expression was increased to an average of 91,470 CD54 molecules per cell.

CD54 expression can be utilized to measure APC activation

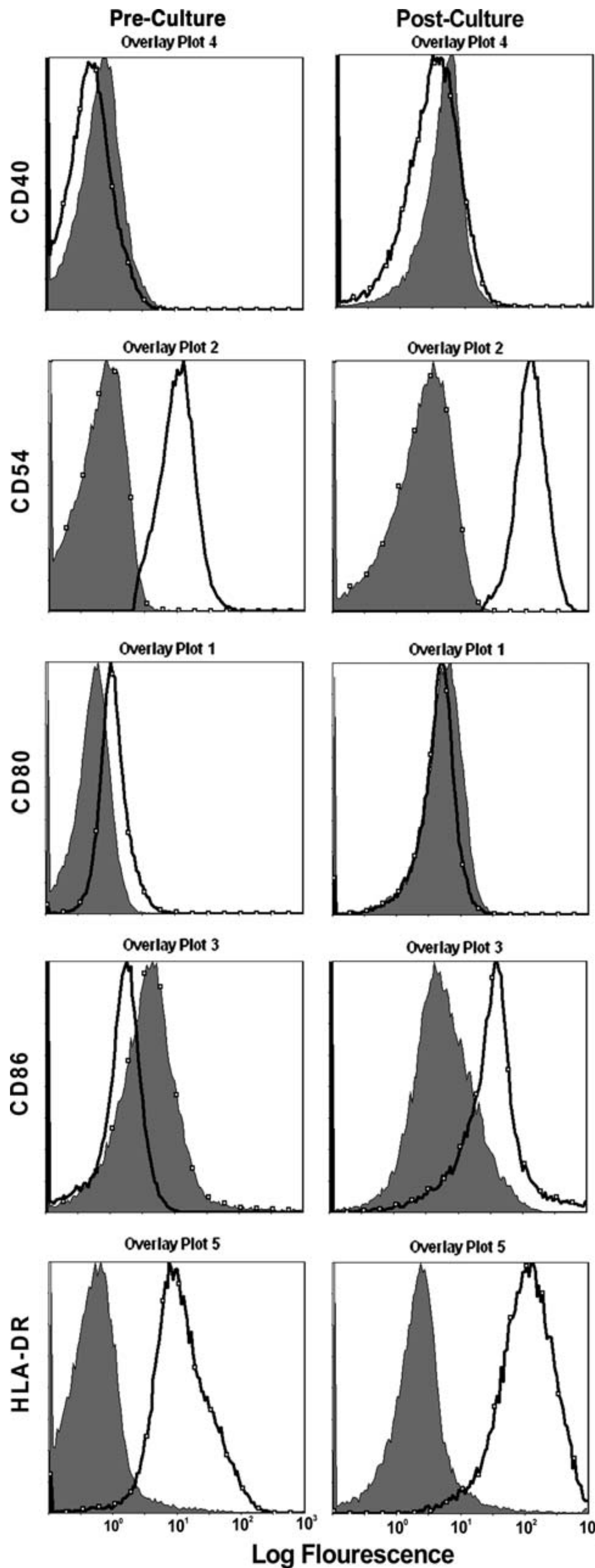
Since GM–CSF has been reported to activate APCs resulting in increased expression of costimulatory molecules and secretion of cytokines [8, 23] we evaluated the expression of costimulatory markers before and after culture. Prior to and immediately after culture with PA2024, cells were stained with antibodies specific for CD54, CD40, CD80, CD86 and HLA-DR. Only CD54+ cells comprised a clearly

Fig. 5 CD54 expression is increased after culture with PA2024 antigen. Cells were incubated with PA2024 for approximately 40 h. Cells were stained with anti-CD80 FITC, CD54 PE, CD86 PE-Cy5, CD40 APC, HLA-DR APC-Cy7 and run on a BD FACSAria where 200,000 events were collected. The shaded peaks represent staining obtained by isotype-matched fluorescent-tagged antibodies and the thick black lines are staining with the surface marker indicated on the *Y* axis for each pair of histograms. Data displayed is from one of seven experiments

delineated population, either before or after culture, whereas this was not the case with the other markers evaluated (Fig. 5). After culture all five markers exhibited varying degrees of increased expression, however only CD54 and HLA-DR expression was substantially and consistently increased after culture. A numerical value was determined for the increase in expression by determining the (MFI) values prior to and after culture and the data are shown as an expression ratio, post-culture MFI/pre-culture MFI in seven independent experiments. The most consistent increase in expression ratios was shown for multiple different donors with CD54 and HLA-DR (Table 1) although CD54 increase had a smaller standard deviation and as previously shown not all HLA-DR+ cells took up antigen. It should be noted that the increase in CD86 expression shown in Fig. 5 is atypical in our experience, whereas the increase in CD54 and HLA-DR is typical and reproducible.

BA7072 is also assimilated by CD54+ cells and CD54 is upregulated as a consequence of culture

To confirm that our observations regarding activation of APCs and the relationship to CD54 expression was not a phenomenon restricted solely to PA2024 we performed further experiments using BA7072, another GM–CSF fusion protein which contains both intra and extracellular sequences of Her2/neu linked to GM–CSF. Cells were cultured with BA7072 spiked with 1% (w/w) FITC labeled BA7072 and then stained for CD54. Figure 6a shows that similar to culture with PA2024, FITC labeled BA7072 uptake is localized to CD54+ cells. In addition, after culture with antigen, cells presented Her2/neu peptides in a dose dependant manner, as assessed by the Her2/neu specific T cell hybridoma Herder (Fig. 6b). In additional experiments we compared the increase in CD54 expression and the costimulatory activity in post-culture cells after culture with either BA7072 or PA2024. Figure 6c shows that culture with BA7072 resulted in similar increases in the upregulation of surface CD54, as assessed by comparing CD54 levels before and after culture, compared to PA2024. In addition the costimulatory activity, as measured in an AlloMLR, induced by culture with either BA7072 or PA2024 (Fig. 6d) was also comparable.



To determine if CD54 upregulation was a phenomenon associated only with activation by GM–CSF, we evaluated TLR ligands for their ability to induce CD54 upregulation.

Table 1 Fold upregulation of CD54, CD40, CD86 and HLA-DR ($n = 7$)

	Median	Mean	Max	Min	SD
CD54	4.9	6.8	10.5	3.6	3.2
CD40	1.2	1.2	1.9	0.4	0.5
CD80	1.1	1.2	2.1	0.3	0.6
CD86	0.6	0.9	2.3	0.4	0.7
HLA-DR	4.9	5.1	7.2	3.1	5.1

CL097 a TLR7 ligand, MPL a TLR4 ligand and finally Poly I:C a TLR3 ligand induced increases in CD54 upregulation comparable to PA2024 (Table 2).

Discussion

Autologous active cellular immunotherapies are an emerging addition to the arsenal for the treatment of cancer. As they are focused upon activating and/or altering the specificities of the immune system, any measure of their relative potency has to be based upon intrinsic immune system properties.

The adaptive arm of the immune system relies upon APCs to activate antigen-specific T cells [1]. This process is achieved by two pathways working in concert: (1) processing and presentation of antigenic fragments via MHC I/II and (2) costimulation via a myriad of molecules, with the most extensive studied being the B7 family [9]. Additionally APCs, such as cells of the monocyte-macrophage family, are also major components of the innate immune response [1, 11]. Thus APC activation can be indicative of the potential to activate both arms of the immune synapse. A pivotal hindrance to assessing APC activation is the lack of a universal measure or assay that can be quantitated. The first step to address this deficiency is the choice of attribute to measure either as a direct indicator of APC activation, for example an increase in an appropriate biological activity such as antigen uptake, presentation or costimulation or an indirect measure such as modulation of surface marker expression.

CD54, by virtue of its biological role, is an appropriate candidate as both a phenotypic marker of APCs as well as an indicator of their activation state. Its primary biological role is to promote cell to cell contact [15], thereby strengthening the interface of the immunological synapse between an APC and a T cell [4]. There is also data indicating that the actual adhesive interaction itself is a costimulatory event [13, 27]. In addition the direct role of CD54 for APC functionality, in terms of activating or priming T cells, is substantiated by a number of murine studies that show an absence of CD54 expression on APCs leads to lowered T

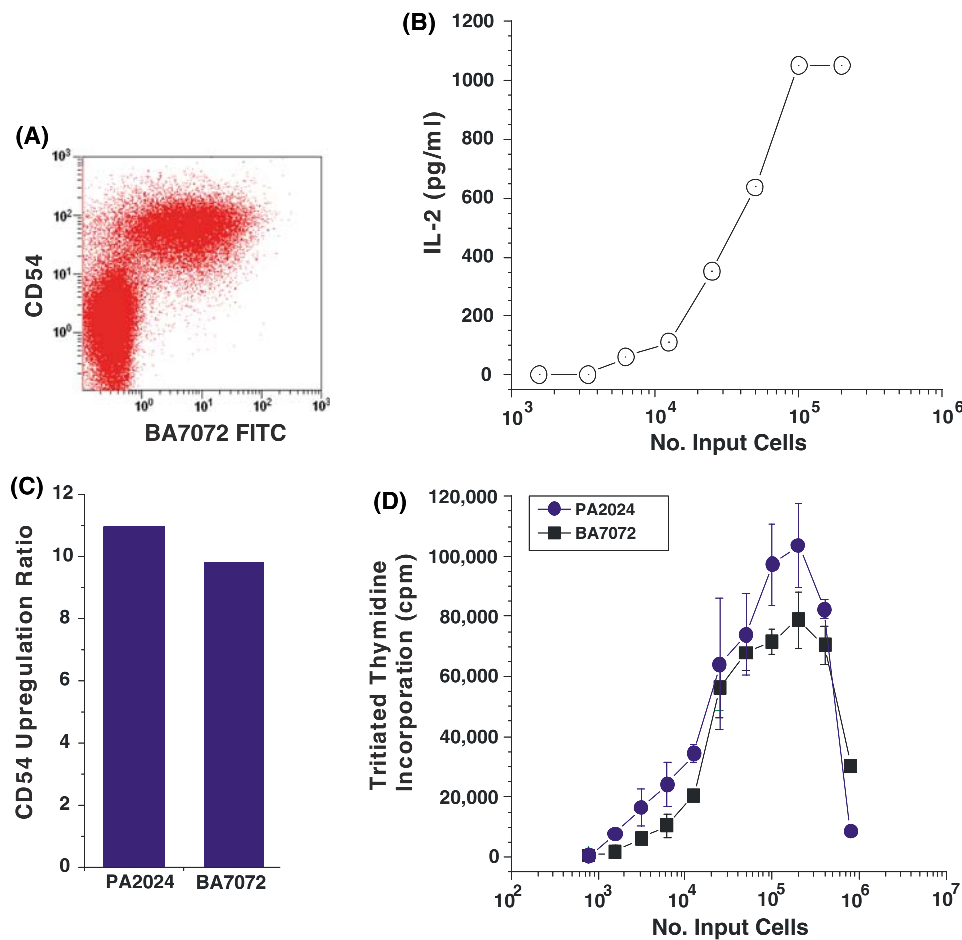


Fig. 6 CD54+ cells take up BA7072 and surface CD54 is upregulated. **a** Cells were incubated with BA7072 spiked with 1% (w/w) FITC labeled BA7072. After approximately 40 h cells were harvested as described in the materials and methods and surface stained for CD54. Stained cells were analyzed on a BD FACSAria™ flow cytometer and data is displayed using CXP software. **b** Cells cultured with BA7072 process and present Her2/neu epitopes to Herder, a Her2/neu-specific T cell hybridoma. As described in Materials and methods, post culture cells were titrated against Herder hybridoma and the level of Herder activation was assessed by measuring the amount of IL-2 produced. **c** CD54 is upregulated after culture with either BA7072 or PA024. Surface CD54 was measured on pre and post culture cells and the relative increase in CD54 expression was reported as a ratio. **d** Cells cultured with either BA7072 or PA2024 possess costimulatory activity. Post culture cells were irradiated and incubated with pooled purified human T cells as described previously and the degree of T cell proliferation was inferred from the amount of tritiated thymidine incorporation

Table 2 CD54 fold upregulation after culture with either PA2024 or TLR3, 4 and 7 ligands

	Experiment #1	Experiment #2	Experiment #3
PA2024	12.6	8.5	14.6
CL097	14.7	5.3	13.1
MPL	16.4	12.0	17.5
Poly I:C	10.4	5.7	15.5

cell activation of naïve T cells [21] or functionally impaired memory T cells [17].

As APCs are composed of different cell types with disparate and distinct physical properties, it is difficult to identify a single quantifiable method to assess APC activation. There is however a common element: all APCs express costimulatory molecules upon activation. Therefore the measure of costimulatory molecule expression or activity is a pertinent correlate of APC activation. While costimulatory activity measured by assays such as the Allo-MLR yield meaningful data and can be used to quantify the degree of activation, this assay system is cumbersome, variable and not suitable as a product release assay.

Our data support the use of CD54 expression as a measure of APC activation for several reasons. CD54+ cells are responsible for uptake, processing and presentation of soluble antigen, which are biological attributes restricted to APCs. In addition, costimulatory activity, an indicator of the activation state of APCs, is localized to CD54+ cells and is enhanced on that cell population after culture. Fur-

thermore as CD54 expression, measured by flow cytometry, is easily distinguished and is consistently increased after culture with antigen, amplifications in CD54 expression can be used as a surrogate measure of APC activation. In comparison with the Allo-MLR assay the CD54 upregulation assay has a low signal to noise ratio. Additionally the upregulation of CD54 is not restricted to a single APC activating moiety, as evidenced by the fact that culture with GM-CSF as well as TLR3, TLR4 and TLR7 ligands all facilitated increases in CD54 expression. It is of note that TLR4 ligands acts through surface binding of TLR4 whereas both TLR3 and TLR7 are expressed in the cytoplasm and thus exert their activation effects through different stimulatory pathways [29]. Thus quantitation of CD54 can be considered a universal measure of APC activation.

In summary APC attributes, such as antigen presentation and costimulatory activity, are restricted to the CD54+ population. The primary requirements for a measure of potency, as detailed by FDA guidelines (FDA/CBER/OCTGT online reference 1) [20], are met by assessing the upregulation of CD54. The methods described here are also practical: upregulation of CD54 can be quantitated rapidly and reliably for all HLA haplotypes. Furthermore, while not a requirement for a potency assay, the reported correlation of CD54 upregulation with survival in clinical trials with sipuleucel-T highlights the biological relevance of potency for this product candidate [manuscript in preparation].

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