Published in final edited form as:

Toxicology. 2009 October 1; 264(1-2): 1–9. doi:10.1016/j.tox.2009.07.021.

A plasmacytoid dendritic cell (CD123+/CD11c-) based assay system to predict contact allergenicity of chemicals

Seyoum Ayehunie, Maureen Snell, Matthew Child, and Mitchell Klausner
MatTek Corporation, Ashland, MA 01721

Abstract

A predictive allergenicity test system for assessing the contact allergenicity of chemicals is needed by the cosmetic and pharmaceutical industry to monitor product safety in the marketplace. Development of such non-animal alternative assay systems for skin sensitization and hazard identification has been pursued by policy makers and regulatory agencies. We investigated whether phenotypic and functional changes to a subset of dendritic cells (DC), plasmacytoid DC (pDC), could be used to identify contact allergens. To achieve this goal, normal human DC were generated from CD34+ progenitor cells and cryopreserved. Frozen DC were thawed and the pDC fraction (CD123+/CD11c-) was harvested using FACS sorting. The pDC were cultured, expanded, and exposed to chemical allergens (N=26) or non-allergens (N=22). Concentrations of each chemical that resulted in >50% viability was determined using FACS analysis of propidium iodide stained cells using pDC from 2-5 donors. Expression of the surface marker, CD86, which has been implicated in dendritic cell maturation, was used as a marker of allergenicity. CD86 expression increased (≥ 1.5 fold) for 25 of 26 allergens (sensitivity = 96%) but did not increase for 19 of 22 non-allergens (specificity = 86%). In a direct comparison to historical data for the regulatory approved, mouse local lymph node assay (LLNA) for 23 allergens and 22 non-allergens, the pDC method had sensitivity and specificity of 96% and 86%, respectively, while the sensitivity and specificity of the LLNA assay was 83% and 82%, respectively. In conclusion, CD86 expression in pDC appears to be a sensitive and specific indicator to identify contact allergenicity. Such an assay method utilizing normal human cells will be useful for high throughput screening of chemicals for allergenicity.

Keywords

Plasmacytoid dendritic cells; allergenicity; CD86; CD34+progenitor cells

Introduction

Langerhans cells (LC)/dendritic cells (DC) represent a heterogeneous group of motile cells including skin and mucosal LC, blood DC, interdigitating cells in the T-cell areas of the lymph node, and interstitial (tissue) DC (Ayehunie et al, 1997). DC monitor “danger” signals in the epidermal, mucosal, and other tissue microenvironments by sampling antigens and transporting them to regional lymph nodes to induce T-cell responses. The activation of DC is considered one of necessary events for the initiation of primary and secondary immune responses (Shi et

Corresponding Author: Seyoum Ayehunie, Senior Scientist, MatTek Corporation, Ashland, MA 01721, Tel: 508-881-6771 ext.213, Fax 508-879-1532; sayehunie@mattek.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

al, 2003). It has also been postulated that chemicals or microbial components provide signals that alert the immune system to initiate appropriate immune responses. These danger signals initiate the immune response and lead to DC activation, maturation, and presentation of antigens to T-lymphocytes.

The use of LC to study contact allergens has been hampered because: 1) LC constitute only 2-4% of the epidermis, 2) LC are difficult to isolate and propagate in vitro, and 3) LC have a short life span in culture. These difficulties have led researchers to use peripheral blood, bone marrow, and umbilical cord blood derived progenitor cells and monocytes derived DC as alternative sources for LC/DC isolation and generation. However, variation in the methodology for generating of LC/DC has led to variability of results.

The utility of utilizing changes in surface marker expression levels (e.g. HLA-DR, CD54, CD80, CD83, and CD86) on LC/DC in the identification of chemical allergens has been investigated by others. For instance, Hulet et al (2005) showed that exposure of monocyte-derived DC to chemical allergens (with slight to moderate effects on cytotoxicity) increased CD86 expression levels but treatment of DC with non-cytotoxic concentrations of chemicals resulted only in modest upregulation of CD86. Ashikaga et al, (2002) also showed increased CD86 expression and enhanced internalization of MHC class II molecules by DCs using THP-1, a human monocyte cell line.

DC can be divided into two distinct subset of cells: 1) myeloid DC (MDC, CD123-/CD11c+) and 2) plasmacytoid DC (pDC, CD123+/CD11c-) based on phenotypic marker expression, ultrastructural features, cytokine responses, and anatomical distribution. However, it is unclear whether the two DC subsets represent a distinct cell lineage or a different state of cell maturation (Summers et al, 2001). While both DC populations can induce strong proliferation of allogeneic naïve CD4+ T-cells (Kadowaski et al, 2000), MDC induce the production of T_H1 type cytokines (IFN- γ and IL-12) and the pDC induce T_H-2 type cytokine (IL-4, IL-10) release. However, others have questioned this view and suggested that pDC are flexible in directing T_H1 or T_H2 responses depending on the nature of stimuli including inflammatory mediators like cytokines, pathogens, or dose of antigens (Celia et al, 2000).

pDC constitute only 0.4% of the peripheral blood mononuclear cell population (Ronnblom and Alm, 2001) and do not express the myeloid lineage marker CD11c. Phenotypically, pDC resemble IgG secreting plasma cells and can be identified by their high expression of the Interleukin 3 receptor alpha chain (CD123) combined with other cell surface markers such as CD45RA, HLA-DR, and CD68 (Farkas et al, 2001). pDC have also been associated with allergic reactions (Jahnsen, et al, 2000). For instance, an increased number of pDC in skin lesions are present in patients with contact allergic reactions such as psoriasis vulgaris (Wollenberg et al, 2002), and Lupus erythematosus (LE) (Farkas L, 2001) suggesting their role in proinflammatory responses. In a short-term homing assays, mDC precursors migrated to peripheral tissues and subsequently to draining lymph nodes (LN), whereas pDC precursors directly enter the LN in an E-selectin dependent manner (Yoneyama, et al, 2004). This finding suggests that the distinct trafficking pathway of pDC to lymph nodes may be important in the initiation of primary immune responses to antigens/chemicals with allergenicity potential.

Since pDC are low expressors of CD86 but are involved in the allergic, inflammatory response, we hypothesized that CD86 expression could be used to develop an allergenicity assay of high sensitivity and specificity. Here, we report the development of a human cell based in vitro pDC assay method for detecting chemical allergens. First, we developed a technology from which a large number of pDC can be generated. Then, we developed a pDC assay protocol which was highly sensitive and specific for identifying contact allergens (n=48 chemicals). We anticipate that the pDC-based assay will prove to be useful for large scale screening of chemicals for their

allergenicity potential. Development of such a non-animal alternative assay system for hazard assessment directly addresses European legislation such as REACH (Registration, Evaluation, and Authorization of Chemicals) and the 7th Amendment to the Cosmetics Directive which significantly limit and or ban animal testing for safety assessment.

Materials and Methods

Generation of DC from CD34+ precursor cells

DC were generated from umbilical cord blood derived CD34+ progenitor cells. Umbilical cord blood was obtained from a local hospital or from the National Disease Research Interchange (NDRI, Philadelphia, PA) following Institutional Board Approval (IRB). Isolation, propagation, and maturation of DC progenitor cells from CD34+ progenitor cells were performed as previously described (Romani et al, 1994; Fraissnette et al 1988, Caux et al, 1992) with modification. Briefly, umbilical cord derived blood mononuclear cells (BMC) were isolated by Ficoll-hypaque density gradient method. Cells were then washed and CD34+ cells were isolated using Dynal magnetic beads (Dynal, Lake Success, NY) following the manufacturer's recommendation. Briefly, BMCs were incubated with blocking reagent and CD34 antibody coated microbeads. Cells were incubated at 4°C for 30 min, washed 2× with buffer, and the cell bead complexes were loaded onto a positive selection column (AS depletion column, Miltenyi Biotech) and placed in a strong magnetic field. The column was washed with buffer to remove cells that were not attached to CD34+ magnetic beads, and then the column was removed from the magnetic source to elute the CD34+ cells. Higher purities of progenitor cells were obtained by loading the CD34+ fraction onto an additional column (MS/RS separation column, Miltenyi Biotech). In this manner, progenitor cells purity in excess of 95% were routinely obtained. To generate dendritic cells, the harvested CD34+ cells were plated into 12-well plates at a density of 1×10^5 cells/well and fed with DC-MM medium (MatTek Corporation). The cells were cultured and expanded for 17 days to generate Langerhans cells/dendritic cells (LC/DC) phenotype. The generated LC/DC were then cryopreserved until further fractionation into pDC.

FACS analysis

To characterize the DC population, cells were washed 2× with wash buffer consisting of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS). Next, cells were re-suspended in cold PBS containing 2% FBS and 2×10^5 cells were seeded into 96-well plates. The appropriate amount of fluorescent dye-conjugated monoclonal antibody (mAb) was added to the respective wells and cells were incubated for 30 min on ice. Finally, cells were washed 2× with wash buffer, fixed with 10% formalin, and analyzed by FACS at the Dana Farber Cancer Institute (Boston, MA) core facility. For each test article, 5000 -10,000 events were collected and analyzed.

Monoclonal Antibodies

Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies to Langerhans cell surface markers CD1a (clone HI149, isotype IgG1), CD40 (clone 5C3, isotype IgG1), CD80 (clone L307.4, isotype IgG1) CD83 (HB15e, isotype IgG1) CD86 (2331(FUN-1), isotype IgG1), HLA-DR (clone G46-6, isotype IgG2a) were purchased from BD Pharmingen (San Diego, CA). CD54 (ICAM 1; Clone BBIGI1, isotype IgG1) were purchased from R&D Systems (Minneapolis, MN). For FACS sorting of myeloid and plasmacytoid dendritic cells, FITC conjugated CD11c (clone KB90, Dako, Carpinteria, CA) and phycoerythrin (PE) conjugated CD123 (clone 7G3; Isotype IgG2a; BD Pharmingen) were used.

Fractionation of DC subsets

DC were washed 2× with wash buffer and 2×10^7 cells were suspended in fresh, cold buffer. To isolate two subsets of DC, pDC (CD123+ / CD11c-) and mDC (CD11c+ / CD123-), the appropriate amount of fluorescent dye-conjugated CD11c and/or PE-conjugated CD123 mAb (10 µl/1 million cells) was added and cells were incubated for 30 min on ice. Labeled cells were then washed 2× with wash buffer, re-suspended in serum free RPMI1640 medium, filtered (mesh size = 30 µm), and volume adjusted to $10\text{--}20 \times 10^6$ cells/ml. Cell sorting was performed using MoFlow (Cytomation, Fort Collins, CO) at the Dana Farber Cancer Institute core facility. The CD123+ CD11c-cells were further expanded in culture using pDC expansion medium (DCP-MM, MatTek Corporation, Ashland, MA) for 10-14 days. The cells were then exposed to test articles

Vehicle selection

For each new test article (TA), the TA was dissolved in three different vehicles, ultrapure water, DMSO, and ethanol. If the TA was soluble in water, water was used as the solvent. If DMSO or ethanol was necessary to solubilize the TA, the final concentration of DMSO or ethanol in the culture medium was $\leq 0.01\%$. At these concentrations, DMSO and ethanol both tested resulted in $> 90\%$ pDC cell viability.

Dose range finding study

Cryopreserved pDC-100 cells were thawed, centrifuged, and transferred to 6-well plates containing pre-warmed assay medium and incubated overnight at 37°C, 5% CO₂. After a 24 hour equilibration period, the cells were re-suspended in fresh medium, counted and cultured in 24-wells at 150K cells/well; different concentrations of test materials and the control were added to wells for an exposure time of 18 hr. Initial dose range finding was performed using 3 concentrations (at 4 fold dilutions starting from 1% of the test article). Tissue viability was measured by the reduction of the vital dye 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) or by FACS analysis following propidium iodide staining. When MTT was used, cell viability was determined from the ratio of MTT optical density (OD) for test chemical treated cells versus the OD for concurrent negative control cells as per the following equation:

$$\text{MTT Tissue viability} = \text{OD (TEST CHEMICAL)} / \text{OD (NEGATIVE CONTROL)} \times 100$$

For viable/dead cell discrimination by FACS analysis, 2 µl of propidium iodide (PI) solution (10 µg/ml) were added to each well containing 2×10^5 cells. Living cells with intact cell membranes exclude PI, while dead cells or cells with compromised membranes allow dye entry. Cells that actively take up the dye fluoresce brightly in the red range of the visible color spectrum. The % of viable cells was quantified using FACS.

If all concentrations used resulted in $<50\%$ viability, additional dose range findings were performed using 3 concentrations of 10 fold dilution below the lowest concentration utilized in the initial dose range finding test. Based on the viability/ dose range data, concentrations that resulted in $>50\%$ viability were selected for allergenicity analysis.

Test articles

To study allergenicity of chemicals and chemical formulations, common contact allergens known to cause allergic contact dermatitis (ACD) were selected from the North American Contact Dermatitis Tray or from published datasets of skin allergens (Gerberick et al, 2004). Both contact allergens (N=26) and non-allergens (N=22) were investigated. Concentrations

that resulted in >50% cell viability following 18 hour incubation at 37°C were used in the various experiments. All test articles were purchased from commercial sources in analytical grade purity either from OmniDERM (Quebec, Canada), ICN Biochemicals (Plainview, N.Y.), and Sigma (St. Louis, MO). For FACS analysis, pDC (1×10^5 cells) were incubated with test material for 18 hr at 37°C. After exposure, the pDC were collected and phenotypic changes were examined by FACS analysis using specific antibodies to CD86.

Statistical analysis

Changes in CD86 expression were quantified by calculating the fold increase (FI), defined as a percentage of CD86 expression of treated pDC divided by the percentage CD86 expression of untreated (control) pDC.

Selection of cut-off value—The test articles were rank ordered according to their level of CD86 expression and these data were compared to historical allergenicity data obtained from LLNA and human testing. A 1.5 fold increase in CD86 expression minimized the differences between sensitivity and specificity of the in vitro and in vivo assay methods. Hence, the cutoff value for allergenicity was set to 1.5 fold increase in order to optimize discrimination between allergens and non-allergens.

As per the recommendations of the ECVAM Biostatistics Task Force (Holzhutter, 1996), the degree of concordance between the in vitro predicted and the in vivo classification were evaluated by calculating the sensitivity, specificity, and accuracy of test results. Data for all 48 test materials were summarized in a 2×2 contingency table (Table 7a). Separate contingency tables for 45 test materials for which LLNA data were available were also constructed (Table 7b and 7c). Contingency tables for the pDC assay were based on predictions from 50% or more of the donors or the total number of assays performed. If a test article is positive for one donor and negative in the second donor, a third donor was used to verify the allergenicity potential of the test article.

Results

Generation of human DC from CD34+ cells derived from umbilical cord blood

A large number of normal human DC were generated from umbilical cord blood CD34+ progenitor cells and cryopreserved. From samples of umbilical cord blood (55-100 ml), 691 ± 582 million DC ($n=16$ donors) were obtained following a 15-18 day culture process. Although the average number of DC generated from an umbilical cord sample was >190 fold more than the starting number of CD34+ progenitor cells, donor to donor variation in cell yield was noted. Overall, the DC number obtained per UBC sample ranged from 280 million to 2.5 billion (Table 1). This result is a significant achievement because previous attempts to develop in vitro assays utilizing normal human DC were hampered by the inability to harvest large numbers of cells (Regnier et al, 1997, Xu, et al 1995). The ability to harvest a large number of dendritic cells from a single donor will aid in assay reproducibility and large scale screening of chemicals for potential allergenicity.

DC Characterization

To characterize the DC population, surface marker expression levels were determined by FACS analysis. Results indicate that the DC express: 1) CD1a (49-70%), HLA-DR (80-92%), as well as co-stimulatory molecules such as CD80 (39-70%), CD40 (37-74%), and CD86 (36-67%), CD206 (20-42%), and low levels of intracellular adhesion molecule-1 (ICAM-1) (22%). Ultrastructural features of the DC were also examined using transmission electron microscopy and the results showed that the DC express both cytoplasmic and plasma membrane bound

Birbeck granules (Figure 1), a unique characteristic of Langerhans cells (Fahrbach et al 2007).

Fractionation and characterization of DC

As shown in Figure 2a, the DC consisted of two distinct subsets, plasmacytoid dendritic cells (CD123+ / CD11c-) and myeloid dendritic cells (CD11c+ / CD123-). The ratio of pDC to mDC averaged 1.2:1. Morphologically, pDC are spherical and of low dendriticity compared to the mDCs which are more dendritic with irregular shape (Figure 2b). pDC had low CD1a expression (12%) while mDC had significant CD1a expression (67%); both cell fractions expressed high levels of HLA-DR (> 70%). Results of surface marker characterization by FACS for DC from a representative donor are shown in Figure 2c.

Expansion of pDC

Some of the previous limitations associated with the use of dendritic cells for allergenicity studies were: 1) short lifespan of cells in culture and 2) insufficient number of DC obtained from a single donor. These limitations were overcome by developing a specific pDC culture medium (DCP-MM, MatTek Corporation, Ashland) that allows expansion and maintenance of pDC in vitro. In the DCP-MM, the cells were maintained in culture for 10-14 days and increased 12-34 fold in cell number. The growth curves of 2 representative pDC preparations are shown in Figure 3.

Functionality of pDC

Since the maturation state of DC is critical for the induction of T cell function (Fahrbach, KM et al., 2007, Lapointe et al 2000, Labeur et al, 1999) and lipopolysaccharide (LPS) and TNF- α are known to induce DC maturation, (Labeur SM et al, 1999), the effect of LPS and TNF- α on pDC surface marker expression was examined. pDC were stimulated with LPS (5 ug/ml) and TNF- α (30 ng/ml) overnight and analyzed using FACS. As shown in Table 2, the expression of the LC-associated co-stimulatory molecules, CD80, CD83, and CD86, increased following exposure to LPS and TNF- α . These results showed that pDCs were functional and responsive to exogenous stimuli.

Allergen/non-allergen treatment of pDC

Preliminary experiments were performed to examine CD86 expression and the chemokine receptor-7 (CCR7; a chemokine receptor expressed by activated DC/LC that is essential for DC-T cell interaction), levels in pDC and mDC following exposure to chemicals with or without allergenicity potential. As shown in Table 3, pDC exposed to allergens showed increased expression of CD86 (Fold increase, FI >1.5); CD86 expression in mDC did not increase following exposure to allergens. Also, non-allergens showed no increase in CD86 expression (FI < 1.5) following exposure to pDC. In contrast, CCR7 expression increased for BB exposure but not for the other allergens or non-allergens (data not shown). Based on these observations, CD86 was deemed a potential marker for allergenicity detection and CCR7 was not further tested.

To further examine the utility of CD86 as a marker for allergenicity, experiments were performed using 4 additional allergens, hydroquinone (HQ), nickel sulfate (NS), cinnamaldehyde (CA), and Balsam of Peru (BP), and 3 non-allergens, propylene glycol (PG), Tween 20 (TW), and ethanol (ET). The results confirmed our previous observations: an FI of >1.5 was obtained for all the allergens but not for the non-allergens (Figure 4) with the exception of Tween-20 (FI = 1.8).

Based on the results of Table 3 and Figure 4, testing of the pDC method was expanded to include a total of 48 test articles (26 allergens and 22 non-allergens). All materials were tested using pDC from a minimum of 2 donors. Data from a representative donor for the 48 test materials are presented in Table 4. Materials were categorized as “allergen” for FI > 1.5 and as “non-allergen” for FI < 1.5. As shown in Table 4, all 25 of the 26 allergens tested gave positive results in the pDC assay (assay sensitivity = 96%) whereas 19 of 22 non-allergens were correctly identified as non-allergens (assay specificity = 86%). Assay results for all 48 test materials are summarized in Table 7a.

Reproducibility

The reproducibility of the pDC assay method was evaluated using the 20 test chemicals shown in Table 5 with pDC obtained from 2-5 different donors. As shown in Table 5, identical assay results were obtained for 16 of 20 test articles regardless of the pDC donor; test results from 1 donor differed from the other donors for the remaining 4 test materials. In total, 81 of 85 (95.3.3%) assay results were identical. Further, the additional 28 test materials shown in Table 4 were tested using pDC from 2 donors, and in all cases, identical results were obtained. Assay-to-assay variability using pDC cells from a single donor for 10 of the test articles was also found to be minimal; no discordant test results were observed (Table 6).

Comparison to historical data from local lymph node assay (LLNA) results

Data for the pDC assay method were compared to historical results from the local lymph node assay, a murine based assay which has been validated as an acceptable alternative to guinea pig tests previously used to predict human allergenicity (Basketter et al, 2003). Historical LLNA data for 45 of the 48 test articles used in pDC assays here reported (Table 4) were available (Basketter et al, 1999, Gerberick, 2004). These data are included in Table 4 and were used to construct contingency tables comparing pDC and LLNA assay results to human allergenicity classifications. As shown in Table 7b & 7c, the sensitivity of the pDC assay was higher than that of the LLNA (96 % vs. 83%) and specificity was similar (86% vs. 82%); overall accuracy of the pDC and LLNA assays was 91% and 82%, respectively. In short, the pDC based assay method gave comparable results to that of the validated animal-based LLNA allergenicity test method and exhibited high levels of sensitivity and accuracy versus historical human test results.

Discussion

Previous reports (Fahrbach et al 2007; Maciejewski-Lenoir, 2006) have shown that CD34+ progenitor cells derived from human umbilical cord blood can be used as a source for large scale production of dendritic cells resembling epidermal LC that contain Birbeck granules (unique marker of LC), CD1a, and Langerin. Production of these cells in larger quantity enabled us to: 1) perform large-scale screening of chemicals for their allergenicity potential, 2) determine intra-assay or inter-assay reproducibility using cells from the same donor, and 3) monitor donor-to-donor variability.

As of 1994, more than 100,000 chemicals were in commercial use and 2,000 new chemicals were introduced every year (Koeter, 1994). Furthermore, the number of new product formulations tested or marketed each year exceeds new chemicals by 10-100 fold. The test methods currently available to assess potential contact allergenicity of materials include: 1) human patch tests, 2) guinea pig maximization test (GPMT), and 3) the murine-based local lymph node assay (LLNA). In GPMT tests, hazard identification is done by visual observations of erythema and edema reactions which are subjective, have difficulties in differentiating between contact allergens and strong irritants, and are time consuming (Silva O.D 1994). Human patch tests are expensive and due to ethical considerations, prescreening with animal

tests is required prior to human clinical studies. Animal tests frequently require the use of Freund's complete adjuvant, which is painful (Magnusson, et al 1969) or the use of radioisotopes. Furthermore, animal tests will be outlawed in Europe as a result of the 7th Amendment to the Cosmetics Directive (Grindon et al 2005) and due to species-to-species variability, animal tests may not accurately predict allergenicity in humans. Therefore, a cost-effective *in vitro* assay system that utilizes cells of human origin to predict contact allergens would be of great benefit for screening of new chemicals and formulations.

Most of the work related to allergenicity testing has focused on the use of animal models such as the guinea pig and mouse. Recently, the murine local lymph node assay (LLNA) has been validated as a stand-alone alternative to guinea pig assays for the identification of skin sensitization hazards (Basketter et al, 2003). The LLNA measures lymphocyte proliferation using incorporation of radiolabeled nucleotides (3H-thymidine or uridine) in draining lymph nodes of mice topically exposed to test chemicals as an indicator of sensitization (Kimber and Weisenberger, 1999). Although the LLNA reduces the number of animals used and reduces the time required to obtain results, it remains an animal based model. In this context, the pDC based assay system here reported gave comparable results to those of LLNA assay while avoiding the use of animals for chemical safety assessment.

While previous studies have investigated the use of CD86 expression by dendritic cells as a marker to identify chemicals with allergenicity potential (Gaspari et al, 1998, Hulette et al, 2005), the use of DC for allergenicity studies was limited in part by the low number of DC isolated from a single donor. Here we report that large number of DC can be generated from CD34⁺ precursor cells from the umbilical cord blood obtained from a single donor. Even though we standardized culture conditions, a large variation in the DC yield from different donors was observed. The reason for this variation is not known but it may be associated with donor-to donor variation similar to what is observed in all biological systems.

Although several researchers have utilized DC for chemical allergenicity studies, the role of CD86 expression level by pDC was not examined. The low level background expression of CD86 by unstimulated pDC makes the pDC-based assay more sensitive and attractive for hazard identification particularly for chemicals with weak allergenicity potential. Here, we have extended earlier studies by specifically focusing on CD123⁺CD11c⁻ dendritic cell subsets for the identification/screening of chemical allergens. Furthermore, we have developed methods to expand pDC (CD123⁺CD11c⁻) and maintain them in culture for extended time periods. Based on an average yield of 700×10^6 unfractionated DC from each umbilical cord blood (UCB) sample (Table 1), DC to pDC expansion factors of 6-20 fold increase in cell number (Figure 2), and a pDC FACS yield of 20%, approximately $1-3.3 \times 10^9$ pDC/UCB sample can be generated. Assuming 1×10^5 pDC are required per assay, $1-3 \times 10^4$ tests would be possible from a single UCB sample. Such a large stock of cells from the same donor would enable large scale screening of chemical allergenicity.

In conclusion, upregulation of CD86 by pDC appears to be a valuable biomarker for chemicals with allergenicity potential. This *in vitro* assay system appears suitable for large-scale screening of chemicals which is difficult to do using existing animal-based methods that requires large number of animals, raise animal welfare concerns, and incur high costs per experiment. The pDC assay method is expected to be cost-effective and, since it utilizes cells of human origin, interspecies extrapolation is avoided. Development and validation of alternative (non-animal) test methods is considered to be a top priority by US and European organizations such as Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the European Center for the Validation of Alternative Methods (ECVAM). In short, increased expression of CD86 by pDC appears to be sensitive and specific marker for the identification of chemicals with allergenicity potential.

Acknowledgments

The research was supported by the National Cancer Institute (NCI) SBIR Grant (#R44 CA106137), National Institute of Health.

References

- Ashikaga T, Hoya M, Itagaki H, Katsumura Y, Aiba S. Evaluation of CD86 expression and MHC Class II molecule Internalization in THP-1 human monocyte derived cells as predictive endpoints for contact sensitization. *Toxicology in Vitro* 2002;16:711–716. [PubMed: 12423654]
- Ayehunie S, Garcia-Zepeda EA, Hoxie JA, Horuk R, Kupper TS, Luster AD, Ruprecht RM. Human immunodeficiency virus-1 entry into purified blood dendritic cells through CC and CXC chemokine coreceptors. *Blood* 1997;90:1379–86. [PubMed: 9269754]
- Basketter DA, Lea LJ, Cooper K, Stocks J, Dickens A, Dearman RJ, Kimber I. Threshold for classification as a skin sensitizer in the local lymph node assay: A statistical evaluation. *Food and Chemical Toxicology* 1999;37:1167–1174. [PubMed: 10654593]
- Basketter DA, Smith Pease CK, Patlewicz GY. Contact allergy: the local lymph node assay for the prediction of hazard and risk. *Clin Exp Dermatol* 2003;28:218–221. [PubMed: 12653718]
- Grindon C, Combes R, Balls M. Toxicity testing: Creating a revolution based on new technologies. *Trends in Biotechnology* 2005;23:299–307. [PubMed: 15922082]
- Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 1992;360:258–261. [PubMed: 1279441]
- Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol* 2000;1:305–310. [PubMed: 11017101]
- Efron PA, Tsujimoto H, Frances R, Bahjat FB, Ungaro R, Debernardis J, Tannahill C, Baker HV, Edwards CK, Moldawer LL. Differential maturation of murine bone-marrow derived dendritic cells with lipopolysaccharide and tumor necrosis factor- α . *J Endotoxin Res* 2005;11:145–160. [PubMed: 15949143]
- Fahrbach KM, Barry SM, Ayehunie S, Lamore S, Klausner M, Hope TJ. Activated CD34-Derived Langerhans Cells Mediate Transinfection with Human Immunodeficiency Virus. *J Virol* 2007;81:6858–6868. [PubMed: 17442711]
- Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (Natural Interferon- α/β -Producing Cells) Accumulate in cutaneous lupus erythematosus lesions. *American J Pathol* 2001;159:237–243.
- de Fraissinette A, Schmitt D, Dezutter-Dambuyant C, Guyotat D, Zabot MT, Thivolet J. Culture of putative Langerhans cell bone marrow precursors: characterization of their phenotype. *Exp Hematol* 1988;16:764–768. [PubMed: 3169159]
- Gaspari AA, Burns R, Nasir A, Ramirez D, Barth RK, Haidaris CG. CD86 (B7-2), but Not CD80 (B7-1), Expression in the epidermis of transgenic mice enhances the immunogenicity of primary cutaneous candida albicans infections. *Infection and Immunity* 1998;66:4440–4449. [PubMed: 9712799]
- Gerberick FG, Ryan C, Kern PS, Dearman RJ, Kimber I, Patlewicz GY, Basketter DA. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* 2004;50:274–288. [PubMed: 15209809]
- Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci* 2004;81:332–343. [PubMed: 15254333]
- Holzhuetter HG, Archer G, Dami N, Lovell DP, Saltelli A, Sjöström M. Recommendations for the application of biostatistical method during the development and validation of alternative toxicological methods. *ECVAM Biostatistics Task Force Report 1. ATLA* 1996;24:511–530.
- Hulette BC, Ryan CA, Gildea LA, Gerberick GF. Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. *Toxicol Appl Pharmacol* 2005;209:159–166. [PubMed: 15885735]

- Jahnsen FL, Lund-Johansen F, Dunne JF, Farkas L, Haye R, Brandtzaeg P. Experimentally Induced Recruitment of Plasmacytoid (CD123^{high}) Dendritic Cells in Human Nasal Allergy. *J Immunol* 2000;165:4062–4068. [PubMed: 11034417]
- Labeur SM, Roters B, Pers P, Mehling A, Luger TA, Schwarz T, Grabbe S. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* 1999;162:168–175. [PubMed: 9886383]
- Labeur M, Roters B, Pers B, Mehling A, Luger TA, Schwarz T, Grabbe S. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* 1999;162:168–75. [PubMed: 9886383]
- Lapointe R, Toso JF, Butts C, Young HA, Hwu P. Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes. *Eur J Immunol* 2000;30:3291–3298. [PubMed: 11093145]
- Kadowakia N, Antonenkoa S, Laub JYM, Yong-Jun Liua YJ. Natural interferon α/β -producing Cells Link Innate and Adaptive Immunity. *J Exp Med* 2000;192:219–225. [PubMed: 10899908]
- Kimber I, Weisenberger C. A murine local lymph node assay for the identification of contact allergens: assay development and results of an initial validation study. *Arch Toxicol* 1989;63:274. [PubMed: 2788401]
- Koeter, HM. What can be expected from *in vitro* methods. In: Rougier, A.; Goldberg, AM.; Maibach, H., editors. *in vitro* Skin Toxicology. Mary Ann Liebert Inc; New York: 1994.
- Magnusson B, Kligman AM. The identification of contact allergens by animal assay, the guinea pig maximization test method. *J Invest Derm* 1969;52:268. [PubMed: 5774356]
- Maciejewski-Lenoir D, Richman JG, Hakak Y, Gaidarov I, Behan DP, Connolly DT. Langerhans cells release prostaglandin D2 in response to nicotinic acid. *J Invest Dermatol* 2006;126:2637–2646. [PubMed: 17008871]
- Régnier M, Staquet MJ, Schmitt D, Schmidt R. Integration of Langerhans cells into a pigmented reconstructed human epidermis. *J Invest Dermatol* 1997;109:510–512. [PubMed: 9326382]
- Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G. Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994;180:83–93. [PubMed: 8006603]
- Ronnblom L, Alm GV. A pivotal role for natural interferon- α -producing cells (plasmacytoid dendritic cells) in the pathogenesis of Lupus. *J Exp Med* 2001;194:F59–F63. [PubMed: 11748288]
- Ryan CA, Gerberick GF, Cruse LW, Basketter DA, Lea L, Blaikie L, Dearman RJ, Warbrick EV, Kimber I. Activity of human contact allergens in the murine local lymph node assay. *Contact Derm* 2000;43:95–102. [PubMed: 10945748]
- Shi Y, James E, Evans JE, Kenneth L, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 2003;425:516–521. [PubMed: 14520412]
- Silva, OD., et al. The ex-vivo local lymph node assay (LLNA). In: Rougier, A.; Goldberg, AM.; Maibach, H., editors. *in vitro* Skin Toxicology. Mary Ann Liebert Inc; New York: 1994.
- Summers KL, Hock BD, McKenzie JL, Hart DNJ. Phenotypic Characterization of Five Dendritic Cell Subsets in Human Tonsils. *Am J Pathol* 2001;159:285–295. [PubMed: 11438475]
- Wollenberg A, Wagner M, Günther S, Towarowski A, Tuma E, Moderer M, Rothenfusser S, Wetzel S, Endres S, Hartmann G. Plasmacytoid dendritic cells; a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *J Invest Dermatol* 2002;119:1096–1102. [PubMed: 12445198]
- Xu S, Ariizumi K, Caceres-Dittmar G, Edelbaum D, Hashimoto K, Bergstresser PR, Takashima A. Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. *J Immunol* 1995;154:2697–2705. [PubMed: 7876542]
- Yoneyama H, Matsuno K, Zhang Y, Nishiwaki T, Kitabatake M, Ueha S, Narumi S, Morikawa S, Ezaki T, Lu B, Gerard C, Shikawa S, Matsushima K. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *International Immunology* 16:915–928. [PubMed: 15159375]

Figure 1.
Representative transmission electron microscopy micrograph showing expression of Birbeck granules in pDC

Figure 2.

FACS sorting and characterization of dendritic cells. **a)** FACS sorting of DC/LC into pDC (CD123+ / CD11c-) and mDC (CD123- / CD11c+); **b)** Morphology of pDC and mDC following a five day culture period. mDC are more dendritic than pDC as observed using light microscopy (40× objective), and **c)** Comparison of surface marker expression of pDC and mDC.

Figure 3.

Expansion of pDC obtained from FACS sorted dendritic cells. pDC were cultured in pDC-MM medium and were counted every 2-3 days. Results of two independent experiments indicate that pDC can be expanded by 12-32 fold.

Figure 4.

FACS analysis showing CD86 expression by pDC following 24 hour exposure to allergens, hydroquinone (HQ), Balsam of Peru (BP), nickel sulfate (Ni), and cinnamaldehyde (CA) and non-allergens, propylene glycol (PG), Tween 20 (TW), and ethanol (ET). After exposure, pDC were washed, labeled with FITC-conjugated anti-CD86 monoclonal antibodies, and analyzed for CD86 expression using FACS. PBS treated cells were used as negative controls.

Table 1

Isolation of CD34+ progenitor cells harvested from umbilical cord blood samples (55-100 ml) and subsequent generation of DC.

Donor #	CD34+ progenitor cells ($\times 10^6$)	Generated DC cells ($\times 10^6$)
722	1	705
740	2	1032
748	1.5	353
753	2.4	488
775	1.4	381
788	0.6	250
796	0.5	249
801	1	500
815	1.5	287
818	1.1	410
819	0.4	472
827	2.4	1506
918	2.2	2500
1014	0.9	830
1037	2	555
1073	0.6	540
Avg. (n=16)	1.3	691.1
St. dev	0.7	582.1

Table 2

FACS data showing surface marker expression by pDC following exposure to LPS (5 µg/ml) + TNF- α (30 ng/ml) for 24 hrs @ 37°C

Surface Marker	Expression (%)	
	PBS	LPS + TNF- α
Control	0.9	1.3
HLA-DR	76.7	93.3
CD80	23.7	59.9
CD83	12.5	43.4
CD86	24.3	50.7

Table 3

CD86 expression and fold increase (FI) in plasmacytoid DC (CD123+ / CD11c-) and myeloid DC (CD123- / CD11c+) cell fractions following exposure to allergens and non-allergens for 24 hr.

Test Article	pDC		mDC	
	CD86 (%)	FI	CD86 (%)	FI
Phosphate buffered saline	12.8	1.0	66.9	1.0
Bandowski's base	71.4	5.6	27.5	0.4
p-Phenylene diamine	28.7	2.2	68.2	1.0
Nickel sulfate	29.2	2.3	83.2	1.2
Dinitrochlorobenzene	20.2	1.6	67.3	1.0
Sodium dodecyl sulfate	12.7	1.0	70.7	1.1
Potassium hydroxide	10.3	0.8	67.3	1.0

Viability and CD86 expression levels by plasmacytoid dendritic cells from a single donor exposed to allergens and non-allergens for 18 hours. Viability was determined by FACS analysis following propidium iodide staining of cells. Allergens are ranked by potency based on the lowest concentration that resulted in ≥ 1.5 fold increase in CD86 expression and $>50\%$ cell viability. Non-allergens are classified based on the highest non-cytotoxic concentration used. Historical data from murine local lymphnode assay (LLNA) and human allergenicity data are included if available.

Table 4

	Test Article	Conc. (%)	Vehicle	Viability (%)	CD86 (%)	Fold Increase	pDC	LLNA	Human/EU
Allergens	1 Tetramethylthiuram disulfide	0.0001	DMSO	85.7	17.6	2.4	+	+	+
	2 Propyl gallate	0.0001	Ethanol	70.6	16.5	1.7	+	+	+
	3 Diphenylmethane diisocyanate	0.0001	Water	90.2	13.7	1.9	+	NT	+
	4 Dinitrochlorobenzene	0.0003	Ethanol	58.2	22.8	2.7	+	+	+
	5 Glutaraldehyde	0.0005	Water	76.5	99.9	11.6	+	+	+
	6 Ammonium hexachloroplatinate (HCPt)	0.001	Water	49.0	41.9	5.7	+	NT	+
	7 p-Phenylene diamine	0.0025	DMSO	88.1	13.2	1.5	+	+	+
	8 Cinnamaldehyde	0.003	Water	69.5	17.9	2.1	+	+	+
	9 Cobalt chloride	0.005	Water	89.6	15.2	1.8	+	+	+
	10 Nickel sulfate	0.006	Water	92.7	12.9	1.5	+	-	+
	11 5-Methyl 2,3 hexanedione	0.01	Ethanol	81.4	14.0	1.9	+	+	+
	12 Fluorescein isothiocyanate	0.01	Water	89.7	99.9	11.6	+	+	+
	13 Beryllium sulfate	0.01	Water	72.6	14.8	1.5	+	+	+
	14 Benzoyl Chloride	0.01	Ethanol	70.0	18.3	1.9	+	+	+
	15 Nickel Chloride	0.01	Water	80.9	43.3	4.5	+	-	+
	16 Chlorpromazine	0.01	Water	55.8	23.0	1.9	+	+	+
	17 Dinitrochlorobenzene sulfonic acid	0.0125	Water	62.1	22.2	2.2	+	+	+
	18 Phenyl benzoate	0.025	Water	68.6	15.8	1.6	+	+	+
	19 Bandowski's base	0.001	DMSO	79.8	16.2	2.1	+	NT	+
	20 Neomycin sulfate	0.1	Water	92.6	32.0	3.7	+	-	+
	21 Chloramine-T	0.5	Water	70.8	97.0	8.1	+	+	+
	22 Penicillin G	1	Ethanol	81.7	21.9	3.0	+	+	+
	23 Ethylene Glycol dimethacrylate	1	Ethanol	64.0	21.9	2.3	+	+	+
	24 Kanamycin	1	Water	89.1		1.9	+	-	+
	25 Hydroquinone	0.0005	Water	89.0	14.2	1.8	+	+	+
	26 Potassium dichromate	0.0002	Water	94.0	9.5	1.0	-	+	+

	Test Article	Conc. (%)	Vehicle	Viability (%)	CD86 (%)	Fold Increase	pDC	LLNA	Human/ EU
27	Nonanoic acid	0.01	DMSO	79.3	47.1	4.9	+	+	-
28	Pyridine	0.01	Water	69.8	17.6	1.5	+	+	-
29	Aluminum Chloride	0.01	Water	89.1	10.5	0.9	-	-	-
30	Ethanol	1	Water	85.1	11.5	1.3	-	-	-
31	Potassium hydroxide	0.025	Water	89.3	9.1	1.1	-	-	-
32	Propylene glycol	0.25	Water	79.7	12.4	1.4	-	-	-
33	Dimethylsulfoxide (DMSO)	0.3	Water	89.6	9.4	1.1	-	-	-
34	Sodium lauryl sulfate	0.0001	Water	76.4	17.0	1.7	+	+	-
35	Zinc sulfate	0.0001	Water	89.5	8.2	0.8	-	-	-
36	Tween 80	0.0001	Water	87.2	10.8	1.1	-	-	-
37	Hydrocortisone	0.0001	Ethanol	83.9	9.2	0.9	-	-	-
38	Glycerol	0.01	Water	86.6	12.0	1.2	-	-	-
39	Hexane	0.01	Water	84.2	12.1	1.2	-	-	-
40	Isopropanol	0.01	Water	86.2	8.4	0.8	-	-	-
41	Phenol	0.001	Ethanol	73.3	8.1	1.1	-	-	-
42	Lead acetate	0.001	DMSO	92.2	6.0	1.0	-	-	-
43	Salicylic acid	0.001	Ethanol	78.2	8.8	0.9	-	-	-
44	Xylene	1	Water	64.1	18.0	1.3	-	+	-
45	Tartaric acid	0.1	Water	89.9	16.6	1.4	-	-	-
46	Manganese chloride	0.1	Ethanol	90.1	11.2	0.9	-	-	-
47	Benzalkonium chloride	0.0001	Water	52.1	5.0	0.7	-	-	-
48	Chlorobenzene	0.01	Water	77.0	14.3	1.0	-	-	-

EU = European Classification

Summary of the pDC allergenicity assay reproducibility for allergens (13) and non-allergens (7) tested using pDC derived from 2-5 donors.

Table 5

		Fold Increase (based on CD86 expression)					
	Test Article	Donor 801	Donor 1037	Donor 827	Donor 787	Donor 855	# positive tests/# donors
Allergens	p-Phenylene diamine	2.8	1.9	1.5	2.5	1.6	5/5
	Dinitrochlorobenzene	5.6	3.3	4.1	4.3	2.7	5/5
	Bandowski's base	6.0	NM	3.9	NM	2.1	3/3
	Nickel sulfate	1.9	2.4	2.3	3.9	4.0	5/5
	Cinnamaldehyde	5.8	2.8	4.8	NM	1.9	4/4
	Hydroquinone	3.2	2.3	2.8	NM	1.8	4/4
	Balsam of Peru	5.5	2.9	4.0	NM	NM	3/3
	Neomycin sulfate	2.3	2.6	1.7	3.0	1.9	5/5
	Fluorescein isothiocyanate	3.0	NM	2.4	3.6	NM	3/3
	Cobalt chloride	1.3*	2.3	1.7	NM	NM	2/3
	Dinitrochlorobenzene sulfonic acid	1.6	2.9	1.7	3.0	2.1	5/5
	Potassium dichromate	1.3*	0.8*	1.0*	1.0*	2.0	1/5
	Glutaraldehyde	NM	NM	NM	4.5	3.1	2/2
Non-allergens	Ethanol	1.1	0.9	1.0	1.0	1.2	0/5
	Sodium lauryl sulfate	1.0	1.0	0.9	1.2	1.8**	1/5
	Potassium hydroxide	0.8	NM	0.8	1.2	1.0	0/4
	Propylene glycol	1.1	0.9	0.7	1.1	1.2	0/5
	Tween 20	1.4	NM	1.5**	0.9	1.1	1/4
	Dimethylsulfoxide	0.8	1.0	1.2	1.0	1.1	0/5
	Phosphate buffered saline	1.0	1.0	1.0	1.0	1.0	0/5

Key: NM = Not measured,

* False negative,

** False positive, Positive test = Fold Increase > 1.5.

Table 6 Assay-to-assay variability observed using pDC cells from a single donor with allergens (5) and non-allergens (5).

	Treatment	Experiment #1				Experiment #2			
		Conc. (%)	Viability (%)	CD86 (%)	FI CD86	Conc. (%)	Viability (%)	CD86 (%)	FI CD86
Allergens	pPDA	0.0025	77.1	32.3	3.1	0.0025	73.3	23.2	2.5
	NISO4	0.012	81.7	41.5	4.0	0.012	77.7	36.3	3.9
		0.006	88.3	25.9	2.5	0.006	83.3	35.8	3.9
	NS	0.1	89.1	32.9	3.2	0.1	96.4	29.8	3.2
		0.05	89.6	30.9	3.0	0.05	96.1	27.9	3.0
	DNBS	0.00625	75	33.6	3.3	0.00625	90.2	15.3	1.7
Non-Allergens	FITC	0.01	88.5	46.9	4.6	0.01	91.2	33.2	3.6
	ETOH	1	87.2	12.3	1.2	1	94.2	9.0	1.0
	SDS	0.0025	88.1	11.6	1.1	0.0025	69.4	10.9	1.2
	KOH	0.025	89	10.6	1.0	0.025	93.4	10.6	1.2
	PG	0.25	87.8	11.3	1.1	0.25	93.6	10.0	1.1
	Tween 20	0.005	89.2	15.2	1.5	0.005	92.7	8.0	0.9
	Phosphate Buffered Saline		87.3	10.3	1.0		90.4	9.2	1.0
	No Antibody Control		87.4	1			88	1	

Table 7

Contingency table for predicting allergenicity comparing; a) pDC and human patch test results (48 test materials), b) pDC and human patch test results (45 test materials), and c) LLNA assay and human patch test results (45 test materials). Results in b) and c) are for the identical set of 45 test materials.

pDC Based Assay	Comparison to Historical Human Data	
In vitro	Allergen	Non-allergen
Allergen	25	3
Non-allergen	1	19
Sensitivity:	0.96	
Specificity:	0.86	
Accuracy:	0.92	
pDC Based Assay	Human	
In vitro	Allergen	Non-allergen
Allergen	22	3
Non-allergen	1	19
Sensitivity:	0.96	
Specificity:	0.86	
Accuracy:	0.91	
	Human	
In vitro/In vivo	Allergen	Non-allergen
Allergen	a	b
Non-allergen	c	d
Sensitivity:	$a/(a + c)$	
Specificity:	$d/(d + b)$	
Accuracy:	$(a + d)/(a + b + c + d)$	
LLNA	Human	
In vivo	Allergen	Non-allergen
Allergen	19	4
Non-allergen	4	18
Sensitivity:	0.83	
Specificity:	0.82	
Accuracy:	0.82	