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CD16 on Dendritic Cells: A Biomarker of Metal Allergens

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Abstract

Biomarkers are commonly used in toxicology for risk assessment that offers distinct and obvious advantages. We constructed a new *in vitro* study model where Dendritic Cells (DC) served as a biomodulator. Differentially expressed surface markers on DC after a 24 hours exposure to medical device relevant metal-allergens and metal-nonallergens were considered as biomarker candidates for the identification and development. Interestingly, we found new functional and dose dependent responses of CD16 on the DCs due to significant down-regulation following exposure to 8 metal-allergens and while the expression remained unchanged when exposed to 10 metal-nonallergens by flow cytometry. The statistical evaluation on CD16 alone yielded p value <0.0001, 88% of sensitivity, 90% of specificity and 89% of accuracy based on receiver operating characteristic (ROC) analysis. We also confirmed that the protein CD86 alone consistently acts as an informative biomarker in prediction of allergenicity for tested materials.

Keywords: Dendritic cell; Metal allergen; Biomarker; CD86; CD16

Introduction

Biomarkers are commonly used in toxicology for risk assessment and clinically as diagnostic and monitoring tests with distinct and obvious advantages [1]. A successful biomarker should be beneficial to address safety concerns, meet the demands required for scientific and regulatory acceptance, and provide an alternative test method to improve protection of people [2,3] while conserving the environment and decreasing animal use i.e., reducing, refining, and replacing the use of animals in research (3Rs) [4-6].

The common test methods for assessing the allergic potential of materials include (1) the guinea pig maximization test (GPMT), (2) the murine-based local lymph node assay (LLNA) and (3) human patch tests. 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 [7]. Human patch tests are not practical in preclinical settings. The LLNA is recommended by international regulatory agencies. However, animal tests, especially with the immune system, may not accurately predict allergenicity in humans due to species-to-species variability. In addition, current science has discovered a mechanism of action that answers why mice do not react to nickel as an allergen, but humans do. Toll-Like Receptor 4 (TLR-4) is an essential mediator in nickel-involved allergic response and hypersensitivity in humans. However, mouse TLR4 signaling was not triggered by nickel due to mouse versions of the TLR4 protein missing a specific amino acid, histidine, in two regions where nickel might bind to and trigger the allergic signaling cascade [8]. The discovery reemphasizes that discrepancies exist between laboratory animals and the human ad hoc immune system.

Since recommended by ICCVAM for over a decade, the LLNA has been acknowledged worldwide as a valid alternative to traditionally accepted guinea pig test methods for assessing Allergic Contact Dermatitis (ACD) hazard potential for most regulatory applications. In 2011, however, ICCVAM noted that only half of the known strong human skin sensitizers can be identified in the LLNA assay (52% or 14 out of 27), all remaining substances require additional testing or information to determine that they are not strong skin sensitizers [9]. Moreover, there is an increase in the incidence of allergy/immunotoxicity-related postmarket adverse events associated with medical devices including metallic alloys, suggesting a safety gap between premarket review and the postmarket surveillance [10-12]. Currently, both the product developers as well as the regulators face challenges in evaluating the immunotoxicity potential of medical devices. There are no human-relevant testing systems available in the non-clinical setting. Only the rodent systems are available for allergen testing. However, as mentioned above, significant difference exists between the immune systems of rodents and humans and thus rodent testing might not predict the allergenic potential of the medical devices in humans.

We initiated this project to identify the clinically relevant biomarkers for predicting human metallic allergens. An *in vitro* cell based assay utilizing human dendritic cells (DCs) was developed. For study model selection, human dendritic cells were chosen for the following features: 1) DCs are potent antigen presenting cells and play a critical role in initiating an immunological signaling cascade while exposed to allergens; 2) DCs are controllers of adaptive immunity that bridge the innate and adaptive immune responses; 3) DCs reside in

lymph nodes, tonsils, bone marrow, peripheral and cord blood, and nasal, thymus, spleen, fetal liver, respiratory, mucosal and skin tissues and constantly sample environmental signals to monitor microbial invasion and chemical exposure; 4) The mechanism of action in LLNA, GPMT, and clinical patch test are all subject to the initiation of DC's functional responses; 5) A commercialized cell source is available with acceptable quality control and reliability [13]; 6) A recent paper has demonstrated the utility of the DC cells in biomarker discovery i.e., CD86 [14]; and 7) Use of human DCs can circumvent animal-based species variation and empower clinical relevance. In addition, the assay is able to integrate a useful statistical method: ROC curve for the sensitivity and specificity analysis that are essential and critical elements in biomarker evaluation. We hypothesize that the alterations of the cell-signaling cascade are mediated by the DCs during antigen processing, and the differences in the cell responses to allergens versus nonallergens are measurable by employing a combination of different techniques. We propose that this DC based bioassay will provide new opportunities to identify allergy-specific biomarkers. Our overall goal of the project is to develop highly sensitive and specific biomarkers for assessing allergenic potential of medical devices in pre-clinical setting. This will aid to diminish the safety gap between premarket review and postmarket surveillance.

Materials and Methods

Cells preparation and mediums

Cryopreserved pDCs (CD123+, CD11c-) and optimized basic and maintenance medium without any antibiotics were purchased from MatTek.com (Cat# DC-100-CRY). The data sheet and specification sheet are available on the website [13] (MatTek Corporation 2012). The handling procedures followed the recommendations of the manufacturer. In brief, the cryopreserved cells were thawed at 37°C water bath and transferred to 15 ml tube to be washed two times by basic medium and seeded into a 15-T flask with 10 mL maintenance medium for overnight. The cells were harvested by centrifugation (300 g for 10 minutes) and seeded into a u-bottom 96-well plate at 2.5×10^4 per well with 50 µL maintenance medium.

Test articles

To study the allergenicity of metallic compounds, the human allergens known to cause allergic contact dermatitis (ACD) and several non-allergens were selected from the ICCVAM database [15] and from published datasets [16]. Both metallic allergens (N=8) and non-allergens (N=10) were investigated (Table 1, Testing compounds).

Metal		Form	Cas No.	Supplier	Purity
Allergen	Platinum	Ammonium hexachloroplatinate (IV)	16919-58-7	Sigma-Aldrich	>99.9%
	Cobalt	Cobalt(II) chloride	7646-79-9	Sigma-Aldrich	>99.9%
	Nickel	Nickel(II) sulfate	10101-98-1	Sigma-Aldrich	>99.9%
	Nickel	Nickel(II) chloride	7718-54-9	Sigma-Aldrich	>99.9%
	Mercury	Mercury(II) chloride	7487-94-7	Sigma-Aldrich	>99.9%
	Beryllium	Beryllium(II) sulfate	7787-56-6	Sigma-Aldrich	≥ 99.0%
	Gold	Gold(I) chloride	10294-29-8	Sigma-Aldrich	>99.0%
	Chromium	Potassium dichromate(VI)	7778-50-9	Sigma-Aldrich	>99.0%
Non-allergen	Aluminum	Aluminum(III) chloride	7446-70-0	Sigma-Aldrich	>99.9%
	Potassium	Potassium hydroxide	1310-58-3	Sigma-Aldrich	>99.9%
	Sodium	Sodium lauryl sulfate	151-21-3	Sigma-Aldrich	≥ 99.0%
	Zinc	Zinc(II) sulfate	7446-20-0	Sigma-Aldrich	>99.9%
	Lead	Lead(IV) acetate	546-67-8	Sigma-Aldrich	>99.9%
	Manganese	Manganese(II) chloride	1/5/7773	Sigma-Aldrich	>99.9%
	Barium	Barium(II) chloride	10326-27-9	Sigma-Aldrich	≥ 99.0%
	Iron	Iron(III) chloride	7705-08-0	Sigma-Aldrich	≥ 97.0%
	Copper	Copper(II) chloride	7447-39-4	Sigma-Aldrich	>99.9%
	Magnesium	Magnesium(II) chloride	7786-30-3	Sigma-Aldrich	≥ 97.0%
Negative control	Hydrocortisone	C21H30O5	50-23-7	Sigma-Aldrich	NA
Positive control	LPS		NA	Sigma-Aldrich	>99.0%

Table 1: Testing compounds.

Testing compounds were listed including LPS as a positive control and hydrocortisone as a negative control. All chemicals were purchased from Sigma-Aldrich with highest possible purity.

The selected concentration of each test article was based on a cutoff of 60% cell viability following 24-hour treatment. All test articles were purchased from Sigma (St. Louis, MO) in analytical grade purity (Table 1). For FACS analysis, pDCs (2.5×10^5 cells per well in 96-well plate) were incubated with the test material for 24 h at 37˚C, in a humidified, 5% CO_2 incubator. After exposure, the pDCs were collected and phenotypic changes were examined by flow cytometric analysis (BD Canto II) using appropriate fluorochrome conjugated monoclonal antibodies to human CDs.

Dose range finding

Cryopreserved pDC-100 cells were thawed, washed twice by warmed basic medium, transferred to 25-T flask containing 10 mL pre-warmed maintenance medium, then incubated overnight at 37˚C, in a humidified incubator at 5% CO_2. Both cell culture media contained no antibiotics and were purchased from and branded by MatTek.com (Ashland, MA). After an overnight equilibration period, the cells were re-suspended in fresh maintenance medium at 3×10^4 cells per well in a U-shape bottom 96-well plate. Test compounds and the controls in basic medium were added to each well-containing cell for 24 hours. Initial dose-range finding was performed using 3 concentrations with 10-fold sequential dilutions starting at 15 mM of each test article. Following 24 hours exposure, the cell viability was determined by staining with 7-Amino-actinomycin D (7AAD) that intercalates into double-stranded nucleic acids in dying and dead cells. The percentage of viable cells was quantified by using (BD Canto II and Diva software) flow cytometry with singlet gating strategy. When any concentration results in viability below 60%, additional dose-range findings were performed using three concentrations of 10-fold sequential dilution below the lowest toxic concentration utilized initially. Based on the dose-range data, concentrations in the well that resulted in greater than 60% viability were selected for further data analysis.

All test compounds were dissolved in endotoxin free pure water predominantly; DMSO and ethanol were used to facilitate dissolution on an as-needed basis. All stock solutions were prepared at 1 or 2 molar and were stored at -20˚C for experimental use. The final concentration of DMSO or ethanol in the culture medium was less than 0.15%. The three sequential doses at 2X concentration of each article were freshly prepared in another 96-well plate and 50 µL was transferred into the cell-contained well with 50 µL maintenance medium in triplicate nature. Thus the final volume was 100 µL per well.

Flow-cytometric procedures and gating strategy

Step A-screening and primary selection: To identify new biomarkers, we focused on the 16 most relevant surface proteins expressed on DC as test candidates following a thorough literature search for primary candidates' selection. First, we exposed the DC to selected compounds *in vitro* and observed the expression of the candidate proteins. Second, we narrowed the number down to five promising targets according to the responsiveness to well-known human metallic allergens and metallic non-allergens. As an initial step, 16 different monoclonal antibodies were examined in one channel (PE) using the same PMT voltage and experimental condition. Following the cell preparation, 19 tubes were prepared and processed in parallel, which include the single-color tube for each monoclonal antibody (16 antibodies) plus the isotype controls (3 isotypes). The following antibodies were used: CD1a (Cat.#561754, Clone HI149, Isotype Ms IgG1, κ), CD16 (Cat.# 560995, Clone 3G8, Isotype Ms IgG1, κ), CD44 (Cat.# 561858, Clone G44-26, Isotype Ms IgG2b, κ), CD54 (Cat.# 560971, Clone HA58, Isotype Ms IgG1, κ), CD56 (Cat.# 561903, Clone B159, Isotype Ms IgG1, κ), CD80 (Cat.# 560925, Clone L307.4, Isotype Ms IgG1, κ), CD83(Cat.# 561959, Clone HB15e, Isotype Ms IgG1, κ), CD86 (Cat.# 560957, Clone 2331 (FUN-1), Isotype Ms IgG1, κ), CD141 (Cat.# 559781, Clone 1A4, Isotype Ms IgG1, κ), CD154 (Cat.# 561720, Clone TRAP1, Isotype Ms IgG1, κ), CD184 (Cat.# 561733, Clone 12G5, Isotype Rat IgG2a, κ), CD197 (Cat.# 561008, Clone 3D12, Isotype Ms IgG2a, κ), CD206 (Cat.# 561763, Clone eB72-1665, Isotype Rat IgG2a, κ),CD208(Cat.#558126, Clone I10-1112, Isotype Ms IgG1, κ), HLA-DR (Cat.#560943, Clone G46-6, isotype Ms IgG2a, κ), TLR-9 (Cat.# 560425, Clone eB72-1665, Isotype Rat IgG2a, κ), corresponding isotype controls (isotype controls of Ms IgG1, κ, Ms IgG2a, κ, Rat IgG2a, κ) were performed accordingly. 7AAD was used as a live/dead stain in this experiment. All fluorochromes and isotype controls were purchased from BD Biosciences Pharmingen (San Diego, CA, USA).

Step B- secondary selection: Five cell surface markers that showed a significant difference in expression between allergen and non-allergen groups were selected from step A and combined in one tube. These antibodies are (PE-Cy7-CD16 (Cat.# 335788, Clone 19.2, Isotype Ms IgG1, κ), V450-CD80 (Cat.# 560442, Clone L307.4, Isotype Ms IgG1, κ), FITC-CD86 (Cat.# 555657, Clone 2331 (FUN-1), Isotype Ms IgG1, κ), PE-CD141 (Cat.# 559781, Clone 1A4, Isotype Ms IgG1, κ), and APC-CD206 (Cat.# 561763, Clone B73.1, Isotype Ms IgG1, κ); plus 7AAD as a live and dead stain.

Flow cytometry staining protocol

Following the exposure period in the 96-well plate, the cells were washed twice with cold staining buffer (BD Cat# 554657), and corresponding antibody panel with appropriate amount of fluorochrome were added to each respective wells and incubated for 30 min on ice. Finally, cells were washed twice with 5%FBS/PBS as a wash buffer and fixed by fixation buffer (BD Cat# 554655).

Flow cytometric analysis

All analyses were performed on a FACSCanto II (Becton Dickinson, CA) flow cytometer. FACS Diva software was used for acquisition. Cytometry setup and tracking beads (CST, BD) were used to initialize PMT settings. Unstained control cells as well as single stained tubes for FITC, PE, PerCP Cy5.5 PE-Cy7, APC, and V450 were prepared and used to setup flow cytometric compensation. In some experiments, rat anti-mouse kappa light chain Comp Beads (BD) were used to set the compensation and were stained according to the manufacturer's instruction. Flow Jo software (Tree Star, Ashland, OR) was used for data analysis and display.

Gating strategy

A doublet exclusion gate based upon (FSC-A vs. FSC-W) was utilized to gate on singlet cells, and then within this gate a second gate using FSC-A vs. SSC-A characteristics was drawn. Using a single color histogram, the isotype controls were used to set the marker. Using these settings, the positivity was determined based upon the isotype controls to exclude the non-specific binding.

Statistical analysis

Changes in CDs' expression were quantified by a percentage of expression in all pDC. For each biomarker, the three replicate measurements were averaged prior to a receiver operating characteristic (ROC) analysis [17]. The ROC curve for a continuous biomarker is the plot of sensitivity against one, minus specificity as the cutoff value varies over the range of all possible values for the given biomarker. An ROC curve illustrates the tradeoff between sensitivity and specificity when choosing a cut-off value for a continuous biomarker, and the area under the curve (AUC) provides an index for the overall predictive performance of the biomarker. For a useless biomarker based on random guess, the ROC curve is the diagonal line in the unit square, and the associated AUC is 0.5. A better biomarker should have a higher ROC curve and a larger AUC. Because LPS and hydrocortisone act as internal controls and are not metals, both were excluded from the ROC analysis.

For CD16 and CD86, we constructed nonparametric ROC curves, estimated the associated AUCs nonparametrically, and performed inference on the AUCs using an asymptotic normal approximation [17]. The results for the two biomarkers were then combined mathematically using a linear discriminant function method [18] and a risk score method [19]. To avoid overfitting in the ROC analyses of the hybrid biomarkers, we adopted a leave-one-out cross-validation approach where for each compound the hybrid biomarker was re-estimated using the remainder of the sample [20]. In the case of hybrid biomarkers, inference on the AUC was based on 1000 bootstrap samples. For each (individual or hybrid) biomarker, we suggested a cut-off value based on the estimated ROC curve to maximize the sum of sensitivity and specificity. For the chosen cut-off value, we then estimated the sensitivity, specificity and accuracy (i.e., the overall rate of correct classification) of each biomarker.

All ROC analyses were conducted using R 2.13.1.

Results

Of the 16 tested markers, DC86, CD80, CD206, CD141 and CD16 were considered as good responders. The expression of CD44 and CD54 in the population of the cells exceeded 95%, was considered saturated and were excluded for the further screening. The other markers showed relative negligible changes relative to comparisons of single dose stimulations of positive and negative controls (Figures 1A and 1B).

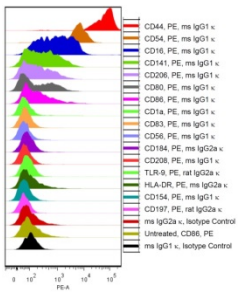

Figure 1A: The (A) shows the expression profile of the 16 PE fluorescent labeled markers on the DC by single dose stimulation of LPS (5 μg/mL) for 24 hours.

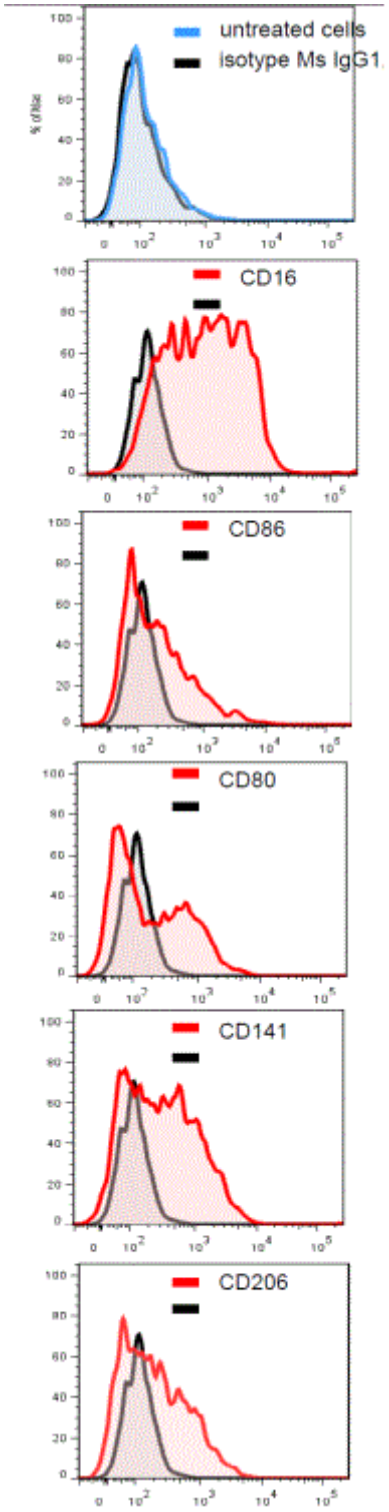

Figure 1B: The black line (B) indicates the expression of isotype control (Ms IgG1, κ), the blue line indicates the basal level of untreated cell in CD86 marker, and the red line indicates the basal level of CD16, CD80, CD141, and CD206 in the same PE channel.

A two-tier selection strategy, primary and secondary selection, has been designed for the biomarker identification. In the primary selection, to screen for meaningful biomarkers in DC-based *in vitro* system and to eliminate unwanted signals from channel-related variation in the flow cytometer, the expression profile of the pre-selected 16 cell surface markers on the DC were measured in a single PE channel. Single dose stimulation of LPS (5 μg/mL) for 24 hours showed DC86, CD80, CD206, CD141 and CD16 to be good responders based on a comparison of single-dose stimulations of positive and negative controls.

In Figure 2, the panels A-C show 5 channel fluorochrome-labeled cell surface receptors from left to right: FITC-CD86, APC-CD206, PE-CD141, PE-Cy7-CD16 and V450-CD80, respectively. The marker expressions on the cells in Figure 2A indicates a comparison of untreated cell (black line) with cells treated by $MgCl_2$ (nonallergen) at dose 1.5 mM (blue line). The Figure 2B indicates a comparison of cells treated by NiSO4 (allergen) at 1.5 mM for 24 hours as pink line with the blue line from cells treated with $MgCl_2$ (non-allergen) at 1.5 mM for 24 hours. Figure 2C indicates a comparison of untreated cells with $NiSO_4$ treated cells.

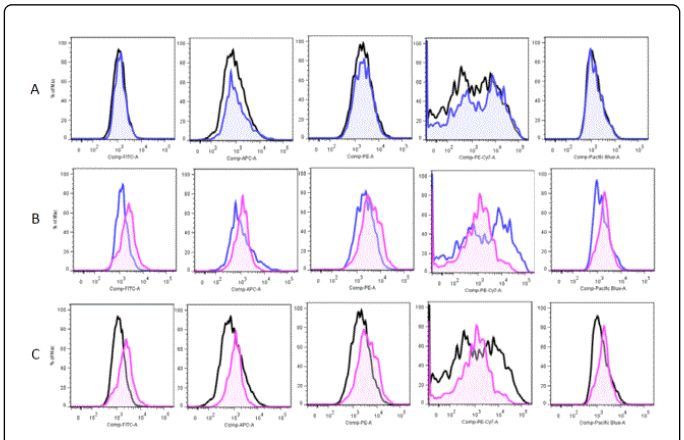

Figure 2: The panels A-C show 5 channel fluorochrome-labeled cell surface receptors from left to right: FITC-CD86, APC-CD206, PE-CD141, PE-Cy7-CD16 and V450-CD80, respectively. The marker expressions on the cells in (A) indicates a comparison of untreated cell (black line) with cells treated by $MgCl_2$ (nonallergen) at dose 1.5 mM (blue line). The (B) indicates a comparison of cells treated by $NiSO_4$ (allergen) at 1.5 mM for 24 hours as pink line with the blue line from cells treated with $MgCl_2$ (non-allergen) at 1.5 mM for 24 hours. The (C) indicates a comparison of untreated cells with $NiSO_4$ treated cells. The histogram of PE-Cy7-CD16 (pink line in 4th column) shifted from right to left (a signal of down-regulation) compared with untreated or nonallergen ($MgCl_2$) treated cells; the histogram of FITC-CD86 in the 1st column was shifted from left to right (a signal of up-regulation) in the panels (B) and (C), respectively. In contrast, there is no obvious shifting in the panel (A) where cells treated by nonallergen $MgCl_2$ (blue line) were compared with untreated cells (black line).

The histogram of PE-Cy7-CD16 (pink line in 4th column) shifted from right to left (a signal of down-regulation) compared with untreated or nonallergen ($MgCl_2$) treated cells; the histogram of FITC-CD86 in the 1st column was shifted from left to right (a signal of up-regulation) in the panels (B) and (C), respectively. In contrast, there is no obvious shifting in the panel (A) where cells treated by nonallergen $MgCl_2$ (blue line) were compared with untreated cells (black line).

In the secondary selection (Figure 3), the cell responses in the five channels were further evaluated against three sequentially reduced doses. CD86 was up-regulated at mid and/or high doses of the allergens (Figure 3A); CD16 was dramatically down-regulated at the high dose and remained at basal levels at the low and mid doses. The doses applied for each allergen appear at a competitive range except $HgCl_2$ due to its higher cytotoxicity. The curve of AuCl dose is not shown because the cytotoxicity exceeds 40% at mid and high doses. The pattern of CD86 up regulation and CD16 down regulation is unique in the allergen panel (Figure 3B) compared with nonallergen panel (Figure 3C) and its clear the biomarker combination is able to differentiate metallic allergens and nonallergens. The cells (Figure 3C) were exposed to nonallergens at the same doses; the CD16 did not show down-regulation despite CD86 up-regulation in $BaCl_2$ and $AlCl_3$ at high dose. CD16 was suppressed in Pb across the three doses. Other data were not shown due to the viability below 60% at the top two doses in the experiment.

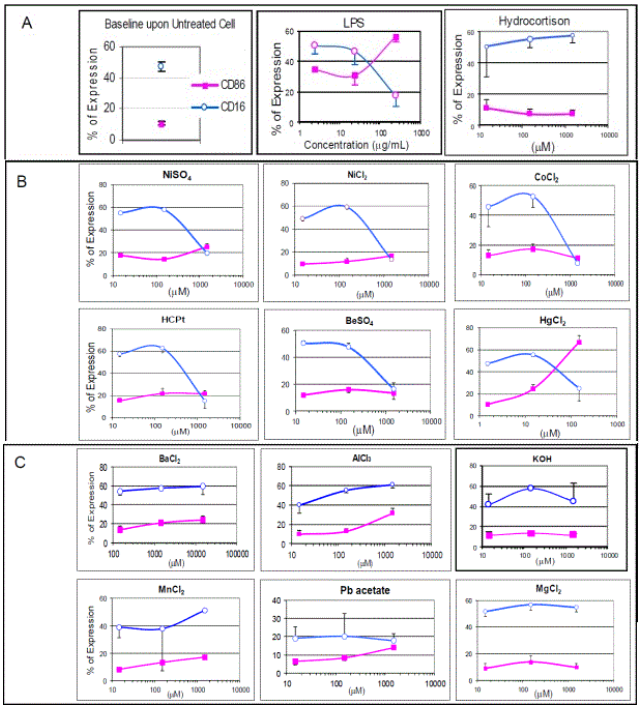

Figure 3: The pink line with solid-square curve represents the CD86 expression and blue line with open circle represents the CD16 expression after 24 hours exposure, respectively. The error bar indicates value of standard error. The y-axis indicates the percentage of the CDs' expressions; the x-axis shows the doses (low, mid and high) with log scale (micromolar). The LPS served as a positive control (microgram per milliliter) and hydrocortisone a negative control (Figure 3A). The dose-dependent curves in Figure 3 represent the typical response pattern of the cells to metallic allergens (Figure 3B) and metallic nonallergens (Figure 3C).

In Figures 4A and 4B, the raw data of CD86 (4A) and CD16 (4B) expression following metallic compound exposure at highest feasible dose (viability greater than 60%) or up to 15 mM were plotted. All

mean value of the triplicate above the cut-off in allergens were counted as true positive, below as false positive; in the nonallergens, above the cut-off were counted as false negative, below as true negative. Results from the ROC analysis indicate a p value of 0.278 and a sensitivity, specificity and accuracy for CD86 alone of 63%, 60% and 61%, respectively. The ROC analysis results a p value of 0.0001; the sensitivity, specificity and accuracy for CD16 alone yields at 88%, 90% and 89%, respectively.

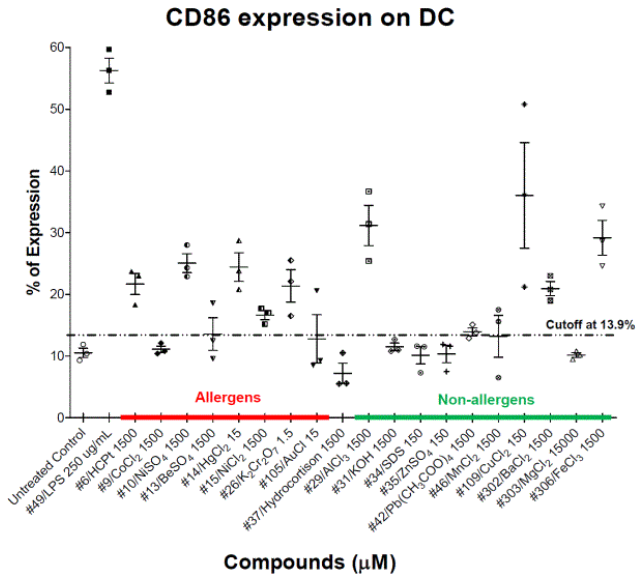

Figure 4A: The Y axis represents the percentage of the expression in the cell population; each point represents readout from flow cytometer in a single well. Each treatment was performed in triplicate (The error bar in each triplicate indicates standard error of mean; the midpoint is a mean value of the triplicate data). The compounds with concentration (micromolar, except LPS was used as microgram per milliliter) in parenthesis were listed on the x-axis. A 13.9% cut-off value describing the optimal separation point between allergens and nonallergens was determined upon statistical maximum sum of accuracy.

All mean values below the cut-off in allergens were counted as true positive allergens, above as false positive; in the nonallergens, above the cutoff were counted as true negative, below as false negative.

The Figure 5 illustrates the estimated ROC curves for the four biomarkers: CD16, CD86, and two hybrid biomarkers based on the risk score (RS) method and the linear discriminant function (LDF) method. Note that the ROC curves for the two hybrid biomarkers overlap to a great extent over the range of 0.1 to 1.0 in the x-axis (i.e., for specificity values less than 90%). Table 2 presents the associated numerical results including AUC estimates, 95% confidence intervals and p-values (for the alternative hypothesis that AUC >0.5), a suggested cut-off value for CD16, CD86, and the corresponding sensitivity, specificity and accuracy estimates.

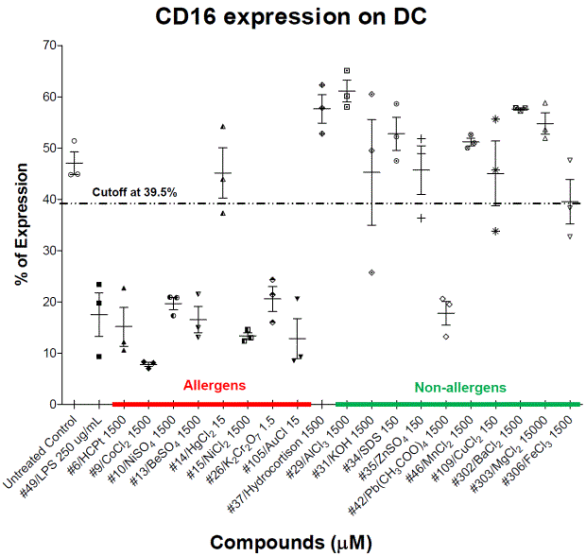

Figure 4B: The Y axis represents the percentage of the expression in the cell population; each point represents readout from flow cytometer in a single well. Each treatment was performed in triplicate (The error bar in each triplicate indicates standard error of mean; the midpoint is a mean value of the triplicate data). The compounds with concentration (micromolar, except LPS was used as microgram per milliliter) in parenthesis were listed on the x-axis. A 39.5% as optimal cut-off value was made upon statistical maximum sum of accuracy (B).

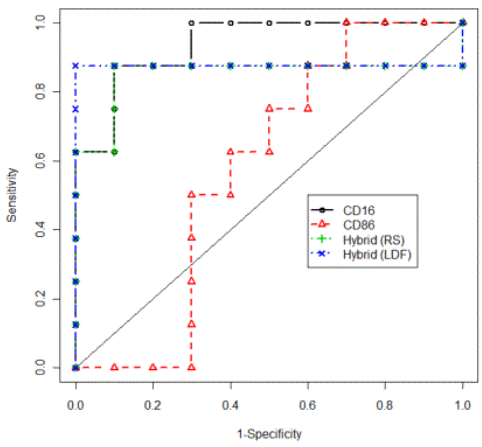

Figure 5: Estimated ROC curves for the four biomarkers: CD16 (solid black line with open circle), CD86 (red dash line with open triangle) and two hybrid biomarkers based on the risk score (RS, green semi-dash line with plus simple) method and the linear discriminant function (LDF, blue dash-dote line with x simple) method. The solid 45 degree line indicates the identity.

As shown in Table 2, numerical results from the ROC analyses

Biomarker	AUC	SE	95% CI		P (AUC>0.5)	Cutoff (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
CD16	0.94	0.04	0.85	1	0	39.5	88	90	89
CD86	0.58	0.13	0.32	0.83	0.2785	13.9	63	60	61
Hybrid (RS)	0.85	0.08	0.74	1	0.001	18	88	90	89
Hybrid (LDF)	0.88	0.1	0.69	1	0.001	18.7	88	100	94

Table 2: Numerical results from the ROC analyses: AUC estimates, standard errors (SE), 95% confidence intervals (CI) and P-values (for the alternative hypothesis that AUC >0.5), as well as a suggested cutoff value for each biomarker and the corresponding sensitivity, specificity and accuracy estimates. The RS and the LDF hybrid biomarker are estimated as CD16-0.54 × CD86 and CD16-0.39 × CD86, respectively.

CD16 performs better than CD86 in both the area under curve (AUC) and in the specificity and accuracy at the chosen cut-off. The two individual biomarkers act in different directions in the sense that CD16 is down regulated by allergens while CD86 is up regulated by allergens. As a result, larger (than cut off) values are indicative of non-allergens for CD16 and allergens for CD86. For both hybrid biomarkers, larger values are indicative of non-allergens. Surprisingly, the hybrid biomarkers do not clearly outperform CD16. The estimated AUC is actually slightly lower for the hybrid biomarkers than that for CD16, though the differences are not statistically significant (two-sided p=0.329 for RS and 0.592 for LDF, based on 1000 bootstrap samples). Although the specificity and accuracy estimates are higher for the LDF hybrid biomarker than that for CD16, the apparent improvement does not clearly achieve statistical significance (two-sided p=0.067, based on 1000 bootstrap samples, without adjusting for the multiplicity due to two hybrid biomarkers being considered) and is likely due to the discreteness in a small sample of compounds.

Discussion

Given the complexity of immunotoxicity and the nature of scientific advancement, we have been seeing more evidence for differences that exist between the animal models and humans with respect to immunotoxicological mechanism of actions. For instance, the mice and humans respond so differently to nickel due to only two amino acid sequence difference of TLR-4 as discovered. The underlying principles of LLNA, GPMT, patch test and ACD are well defined where Langerhans cells (LCs), immature skin dendritic cells (DCs), play a central role in initiating allergic responses in T-cell-mediated delayed-type hypersensitivity/allergy. While substance is up taken by skin DCs, the cells undergo a maturation process and migrate to the draining lymph node for presenting processed substance, hapten, to T-cells. If the hapten is recognized and represented as an allergen, then the T-cells are activated toward differentiation and proliferation. When the hapten appears again, the activated T-cell reacts with it and elicits an allergenic response. The degree of the T-cell proliferation is measured and calculated as stimulate index (SI) in LLNA. A higher SI (greater than 3 as a standard cut-off) indicates more T-cell proliferation that is usually translated as an allergen, vice versa as a nonallergen.

Here, human DCs are employed as an immunomodulator for the biomarker discovery. While the cells were stimulated by metallic allergens or nonallergens, the differences of functional responses/signaling cascade from the cells were carefully detected by measuring cell surface protein/CDs expression with well-designed internal controls throughout the assay for monitoring the testing system and ensuring the cells' functionality. Statistical evaluations were performed for the biomarkers in the two elements, sensitivity and specificity, by ROC curve analysis. In the previous report [14], the CD86 on the pDC (the same cell source) was confirmed to be serving as a biomarker for prediction of human allergens. We integrated the CD86 as a reference for comparison of a newly identified biomarker's performance.

To investigate the capability of the cell as to whether or not it would perform as a meaningful immunomodulator, the functional profiles of the cell were measured following exposure to well-known human allergens and non-allergens in a two tier-selection strategy. We used a single channel PE for the primary selection. The use of a single-channel strategy helps eliminate nonspecific signals that may be generated among the channels and enhance the confidence in choosing the selected markers. Following the exposure, the primary selection was intended to screen out significant changes among the candidates, minimal changes were excluded. After the primary screening, five markers based on the significant responses in the first tier were further tested against a three-sequential-dilution-dose exposure where the DCs were exposed to 8 well-known human metallic-allergens and 10 well-known human metallic-nonallergens with internal untreated, positive and negative controls for monitoring the integrity of the assay. The testing system is also able to normalize the changes into fold changes to eliminate the cell and laboratory-based variations. The results indicated that CD86 showed a degree of up-regulation following allergen stimulation in the ROC curve analysis in this assay, which is consistent with the previous published report [14]. Moreover, the CD16 appears a much better performer than LLNA in sensitivity and specificity when exposed to metallic allergens and nonallergens. We also observed that the allergenic response of $NiSO_4$ is similar to $NiCl_2$ in the system.

In evaluating the performance of LLNA in prediction of metallic allergens, two of five human allergens were noted as negative i.e., Be, which causes cell-mediated allergy in the lung, and Ni, which causes allergy in the skin, both remained negative [21]. The data indicate that 60% of sensitivity was achieved. There were no nonallergens incorporated into the study, and the specificity information appears unknown. Here, CD16 in the *in vitro* testing model performed with 88% sensitivity, 90% specificity and 89% accuracy and thus works much better than that of LLNA. In addition, ICCVAM in 2011 noted that since only half of the known strong human skin sensitizers can be identified in the LLNA assay (52% or 14 out of 27), all remaining substances require additional testing or information to determine that they are not strong skin sensitizers [9].

Many cell phenotypic markers are involved in immune responses. However, capability of CDs on DC in indication of metal allergens remains largely unknown. CD86 is a cell surface receptor/protein

expressed on many antigen-present cells (APCs) and plays an important role in CD28 on T-cell mediated signaling co-stimulate pathway for T cell activation and survival. CD86 works in tandem with CD80 to prime T-cells. Although CD86 was reported to be capable of indicating allergens [14], we confirm that CD86 could be considered as a biomarker of metallic allergens.

CD16 are also found on the surface of many other immune cells e.g., natural killer cells, neutrophil polymorph nuclear leukocytes, monocytes and macrophages [22], reemphasizing APCs share as apart from their characteristic feature and functionality in systemic immunity. It is involved in allergy diseases [23], but the mechanisms of signaling transduction remains unclear. Interestingly, the new function of CD16 on DC as a biomarker in the indication of metal allergens has not been previously reported. Further mechanistic investigation may be needed. This study demonstrated a superior performance of CD16 alone in terms of sensitivity and specificity in predicting metallic allergens.

Furthermore, a mathematical hybridization method unfolded a new approach that may improve the analysis of the performance of a group of biomarkers compared to each individually. A synthetic effect was observed in our pilot studies where multi-selected biomarkers were hybridized by giving equal weight, suggesting that a panel of biomarkers gives a more powerful prediction of human allergens, including some metals, than that of any biomarker response taken individually (data not shown). The advantage of the combination may enhance the statistical confidence when multiple biomarkers are mathematically hybridized to predict single chemical allergenic potential. We, unfortunately, have seen minimal improvement of the two biomarkers hybridized in this study, where CD86 and CD16 were hybridized mathematically, likely due to a small number of the testing samples. Further studies will be conducted to confirm this finding.

Human allergen classification

Certain metal are capable of causing immunological sensitization and subsequent allergic disease in sensitized individuals. The skin is most often the target organ with allergic contact dermatitis (ACD)/ allergy/hypersensitivity. The 18 metallic compounds include 8 well-known human allergens and 10 well-known nonallergens based on the European Union (EU), World Health Organization, CDC and ICCVAM classifications, were assigned for the biomarker identification. Nickel, mercury, cobalt, chromium, gold, beryllium and platinum are listed as human allergens, whereas, aluminum and copper seem to be controversial. Both were classified as non-allergens in the ICCVAM database. We have seen CD86 increases in both, despite CD16 showing no significant down regulation. The incidence of the formerly almost non-existent aluminum allergy has increased with the number of vaccinations given [24].

Although copper is one of the essential metals needed for humans to maintain healthy, clinical data support a copper-nickel cross-reactivity concept [25]. Copper is used in a very wide variety of different applications to which many people are exposed every day. Despite this, copper allergies are not very common as an allergen in general population, a low allergic incidence such as with intrauterine devices (IUDs) has been considered a cause of copper allergies [26,27]. In copper-treated DCs, the degree of up-regulated CD86 and down-regulated CD16 shows a change between that of allergens and nonallergens.

In summary, the data indicate that a new functionality of CD16 shows promise for use as a preclinical biomarker for screening potential allergenic responses to metal containing devices. CD16 alone is able to determine allergens from nonallergens statistically. The overall performances of selected biomarker, CD16 is superior to that of LLNA in predicting the metallic allergens. The non-animal DC-based study model demonstrated the utility of this *in vitro* assay in the biomarker identifications. The biomarker identification and development reveals a future direction in addressing current preclinical and regulatory challenges.

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