CD16 on Dendritic Cells: A Biomarker of Metal Allergens

Ying Mu1*, Athena Keene2, Heba Degheidy3, Zhiwei Zhang4, Catherine Li5, Chandramallika Ghosh1, James Weaver2 and Marilyn Lightfoote1

1Center for Devices and Radiological Health, U.S. Food and Drug Administration, Silver Spring, USA
2Alton Chemical Corporation, Richmond, USA
3Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, USA
4University of California, Riverside, Riverside, USA
5Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, USA

*Corresponding author: Ying Mu, Center for Devices and Radiological Health, US Food and Drug Administration, Silver Spring, USA, Tel: +724-759-3066; Fax: +301-796-9826; E-mail: ying.mu@fda.hhs.gov

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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, 86% of sensitivity, 90% of specificity and 90% 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 animal tests. In GPMT tests, hazard identification was done by visual observations of erythema and edema reactions, which are subjective, 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 DCs' 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 | 10101-96-1 | Sigma-Aldrich | >99.9% |
| Nickel | Nickel(III) 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 | Sigma-Aldrich | NA |
| Positive control | | | | |

**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% CO₂ 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 CD's.

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% CO₂. 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 × 10^8 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 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 promisingtargets 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 G4-26, 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 HB115e, Isotype Ms IgG1, κ), CD86 (Cat.# 560957, Clone 2331 (FUN-1), Isotype Ms IgG1, κ), CD11c (Cat.# 559781, Clone I4, 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 IIo-1112, Isotype Ms IgG1, κ), HLA-DR (Cat.#560943, Clone G4e-6, Isotype Ms IgG2a, κ), TLR-9 (Cat.# 564025, 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-CD114 (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 washing 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 FACS Canto 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 MgCl₂ (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 MgCl₂ (non-allergen) at 1.5 mM for 24 hours. Figure 2C indicates a comparison of untreated cells with NiSO₄ 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 (MgCl₂) 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 MgCl₂ (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 HgCl₂ due to its higher cytotoxicity. The curve of AuCl₃ 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 BaCl₂ and AlCl₃ 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 2](image-url)

**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 MgCl₂ (nonallergen) at dose 1.5 mM (blue line). The (B) 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 MgCl₂ (non-allergen) at 1.5 mM for 24 hours. The (C) indicates a comparison of untreated cells with NiSO₄ 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 (MgCl₂) 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 MgCl₂ (blue line) were compared with untreated cells (black line).

![Figure 3](image-url)

**Figure 3**: The pink line with solid-square 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 viability (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 negative; 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).

As shown in Table 2, numerical results from the ROC analyses
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, specificity and accuracy, 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 NiSO₄ is similar to NiCl₂ 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 allergic 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 intradermal 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 allergic 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.

References

1. Biomarkers Definitions Working Group (2001) Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clin Pharmacol Ther 69: 89-95.
2. Rhomberg LR (2010) Toxicity testing in the 21st century: how will it affect risk assessment? J Toxicol Environ Health B Crit Rev 13: 361-375.
3. Zhang Q, Gamboa da CG, Von Tungeln LS, Jacob CC, Brown RP, et al. (2012) Urinary biomarker detection of melamine- and cyanuric Acid-induced kidney injury in rats. Toxicol Sci 129: 1-8.
4. Balls M (2009) Replacing, reducing and refining animal experiments: Making sound progress, but must do better. Altern Lab Anim 37: 579-580.
5. Cotton P (1993) Animals and science benefit from replace, reduce, refine effort. JAMA 270: 2905-2907.
6. Gad SC (1990) Recent developments in replacing, reducing, and refining animal use in toxicologic research and testing. Fundam Appl Toxicol 15: 8-16.
7. Basketter DA, Scholes EW (1992) Comparison of the local lymph-node assay with the guinea-pig maximization test for the detection of a range of contact allergens. Food Chem Toxicol 30: 65-69.
8. Schmidt M, Raghavan B, Muller V, Vogl T, Fejer G, et al. (2010) Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. Nature Immunology 11: 814-819.
9. Birnbaum LS (2011) NIEHS response to ICCVAM test recommendations relevant to allergic contact dermatitis (ACD) hazard testing and identification of strong sensitizers.
10. Wizemann T (2011) Public health effectiveness of the FDA 510(k) clearance process: Measuring postmarket performance and other select topics: Workshop report.
11. Zeng Y, Feng W (2013) Metal allergy in patients with total hip replacement: A review. J Int Med Res 41: 247-252.
12. Dahms K, Sharkova Y, Heitland P, Pankuweit S, Schaefer JR (2014) Cobalt intoxication diagnosed with the help of Dr House. Lancet 383: 574.
13. MatTek Corporation (2012) Dendritic (langerhans) cells.
14. Ayehunie S, Snell M, Child M, Klausner M (2009) A plasmacytoid dendritic cell (CD123+/CD11c-) based assay system to predict contact allergenicity of chemicals. Toxicology 264: 1-9.
15. No Authors Listed (2012) Use of the murine local lymph node assay for potency categorization.
16. Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, et al. (2004) A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermatitis 50: 274-288.
17. Zhou XH, Obuchowski NA, McClish DK (2002) Statistical methods in diagnostic medicine. John Wiley & Sons, New York.
18. Su JQ, Liu JS (1993) Linear-combinations of multiple diagnostic markers. JAMA 270: 2905-2907.
19. McIntosh MW, Pepe MS (2002) Combining several screening tests: Potential. We, unfortunately, have seen minimal improvement of the 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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22. Janeway C (2001) Appendix II. CD antigens. In Immunobiology (5th edn). Garland, New York.
23. Davoine F, Lavigne S, Chakir J, Ferland C, Boulay ME, et al. (2002) Expression of Fc gamma RIII (CD16) on human peripheral blood eosinophils increases in allergic conditions. J Allergy Clin Immunol 109: 463-469.
24. Baker SL (2011) Vaccinations and allergy shots causing allergies to aluminum.
25. Wohrl S, Hemmer W, Focke M, Gotz M, Jarisch R (2001) Copper allergy revisited. J Am Acad Dermatol 45: 863-870.
26. Cao BM, Zheng YD, Xi TF, Zhang CC, Song WH, et al. (2012) Concentration-dependent cytotoxicity of copper ions on mouse fibroblasts in vitro effects of copper ion release from TCu380A vs TCu220C intra-uterine devices. Biomed Microdevices 14: 709-720.
27. Gobba F, Bianchi N, Verga P, Contessa G, Rossi P (2012) Menometrorrhagia in magnetic resonance imaging operators with copper intrauterine contraceptive devices (Iuds): A case report. Int J Occup Med Environ Health 25: 97-102.