

The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test – human cell line activation test (h-CLAT)

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Abstract Recent regulations for cosmetics in Europe prohibit animal testing for evaluating the sensitization potential of chemicals to improve animal welfare. Yet, there is not an acceptable Organization for Economic Co-operation and Development non-animal skin sensitization test method. Several in vitro skin sensitization methods that focus on the activation of Langerhans cells, including human cell lines, are being evaluated as possible alternatives. In our previous study, we optimized our human cell line activation test (h-CLAT) using THP-1 cells (monocytic leukemia cell line) and conducted an inter-laboratory study. We found that measuring CD86/CD54 expression may be useful for predicting skin sensitization. The aim of this study was to confirm the relationship between CD86/CD54 expression and THP-1 cell viability in the h-CLAT. In this study, 21 allergens (e.g., dinitrochlorobenzene, *p*-phenylenediamine, Ni) and 8 non-allergens (e.g., SLS, lactic acid) were evaluated. For each chemical,

more than 10 concentrations that gave a predicted cell viability range of 20–95% were used. The data showed that expression patterns of CD86/CD54 differed depending on chemical. For most allergens, cytotoxicity (65–90% cell viability) was needed for enhancement of CD86/CD54 expression. The criteria of “CD86 $\geq$ 150 or CD54 $\geq$ 200” resulted in an accuracy of 93%, which confirms appropriate cut-off criteria for h-CLAT. Furthermore, a good correlation was observed between EC3 of local lymph node assay and EC150 (CD86) or EC200 (CD54) of h-CLAT (12 or 16 chemicals, respectively), which would provide a useful estimate of allergic potency. These findings suggest that h-CLAT would be a good robust in vitro skin sensitization test.

Keywords THP-1 · h-CLAT · CD86 · CD54 ·
In vitro skin sensitization · Cell viability

Abbreviations

CV75	cell viability 75% concentration
DC	dendritic cells
DMSO	dimethyl sulfoxide
EC3	estimated concentration that yield a three-fold stimulation
h-CLAT	human cell line activation Test
HTD	highest technical dose
IC50	inhibited cell viability to 50%
LC	Langerhans cell

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LLNA	local lymph node assay
MFI	(Geometric) mean fluorescence intensity
OECD	Organization for Economic Cooperation and Development
PI	propidium iodide
RFI	relative fluorescence intensity
SD	standard deviation

Introduction

In the development of personal care products, such as cosmetics that are used on the skin directly, the evaluation of the skin sensitization potential of the components of the product is extremely important. Guinea pig methods (e.g., guinea pig maximization test) have been the main methods used. More recently, the local lymph node assay (LLNA), as part of a quantitative risk assessment process, was proposed as an early evaluation tool (Gerberick et al. 2001). Soon after, Gerberick et al. (2004) provided a database of 211 chemicals. However, enforcement of a new cosmetics law (7th Amendment to the Cosmetic Directives) in Europe or a greater emphasis of animal welfare in the world will make the use of animals in the evaluation of skin sensitization more difficult in the future, especially for the development of cosmetics.

Under these situations, several non-animal skin sensitization test methods have been proposed recently (Basketter et al. 2005), but up to now, an authorized in vitro test, like the Organization for Economic Cooperation and Development (OECD) test guideline, has not been developed. Because the mechanism of skin sensitization is too complex and it is very hard to reproduce all mechanisms in one in vitro system, the development of an in vitro method should consider or address various aspects of the sensitization process (e.g., the potential dermal penetration of a chemical, the protein/peptide interaction, and the initiation of an antigen-specific immune response; Basketter et al. 2005). The availability of these methods has led to the suggestion that a tiered approach using a battery of these methods could be used as an alternate to the in vivo skin sensitization method (Aptula et al. 2006).

During the induction phase of skin sensitization, Langerhans cells (LC) play a critical role because of their ability to initiate immune responses by processing and presenting antigens after exposure to chemical allergens. Upon antigen capture, LC differentiate, mature,

and migrate to the draining lymph nodes where they present the allergens to naive T cells and trigger their proliferation (Hart 1997; Banchereau and Steinman 1998). During the course, LC maturation is characterized by the upregulation of major histocompatibility complex (MHC) class II and expression of co-stimulatory molecules, such as CD86, CD54, CD80, and CD40 (Aiba and Katz 1990; Ozawa et al. 1996) as well as cytokine production like interleukin (IL)-1 β (Enk and Katz 1992). Many of the antigen-specific immune response methods have used dendritic cells (DC) including LC (Ryan et al. 2001). The DC were mainly derived from human peripheral blood mononuclear cell or CD34⁺ hematopoietic progenitor cell obtained from human cord blood. Several investigators have published some promising data using these cells as surrogates for LC (Aiba et al. 1997, 2000; De Smedt et al. 2002; Aebly et al. 2004; Staquet et al. 2004), but there are still some technical issues for the routine use of these cells in in vitro skin sensitization tests. The technical issues are donor to donor variability and availability of human blood (Aiba et al. 1997; Rougier et al. 2000).

To avoid the issues with obtaining DCs, some human cell lines, THP-1 cells, U937 cells (human histiocytic lymphoma cell line), KG-1 cells (human myeloid leukemia cell line), and MUTZ-3 (human monocytic cell line), are used (Ashikaga et al. 2002; Yoshida et al. 2003; Rousset et al. 2002; Hulette et al. 2002; Ade et al. 2006; Azam et al. 2006). Previously, we have developed an in vitro skin sensitization test based on alteration of CD86 expression and internalization of MHC class II using THP-1 cells (Ashikaga et al. 2002) and alteration of CD86/CD54 expression using THP-1 cells and KG-1 cells (Yoshida et al. 2003). More recently, we optimized the in vitro skin sensitization test using THP-1 cells and reported that the assay, the human cell line activation test (h-CLAT), could predict various allergens with high reproducibility between two facilities (Ashikaga et al. 2006; Sakaguchi et al. 2006).

However, the relation between CD86/CD54 expression and concentration of test chemicals (dose response) has not been fully characterized. In the h-CLAT, the cutoff value of CD86/CD54 expression determines the chemical to be positive/negative, but the dose setting is also critical for level of CD86/CD54 expression. Only after evaluating the dose response for a variety of allergens will the role of test concentration be better understood. In addition, the relationship between minimum applied test dose for

augmentation of CD86/CD54 expression and allergic potency could be obtained from this dose response study. The work presented in this article used 29 chemicals (21 allergens and 8 non-allergens) to clarify and better understand the relationship between CD86/CD54 expression and THP-1 cell viability.

Materials and methods

Cells and medium

THP-1 cells from American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 0.05 mM 2-mercaptoethanol, and 1% of antibiotic-antimycotic (Invitrogen). Passage number of the THP-1 cells was monitored, and new aliquot of the same lot number was set up every 2 months to minimize variability. Cell morphology and replication rate remained constant during and between the 2-month periods of each new aliquot of the same lot number.

Chemicals

Twenty-one allergens employed in the experiment were *p*-benzoquinone (p-BQ), 1-benzoylacetone (1-BA), dinitrochlorobenzene (DNCB), potassium dichromate (PD), glutaraldehyde (GA), hydroquinone (HQ), *p*-phenylenediamine (PPD), propyl gallate (PG), formaldehyde (FA), isoeugenol (IE), 2-mercaptobenzothiazole (2-MBT), ethylene diamine (ED), cinnamaldehyde (CA), phenylacetaldehyde (PAA), nickel sulfate hexahydrate (Ni), resorcinol (RC), α -hexylcinnamaldehyde (HCA), eugenol (EU), linal (LI), pyridine (Py), and ammonium tetrachloroplatinate (Pt). The eight non-allergens were acetanilide (AA), benzalkonium chloride (BZC), glycerol (Gc), 4-hydroxybenzoic acid (4-HBA), lactic acid (LA), methyl-4-hydroxybenzoate (methylparaben; MP), vanillin (VA), and sodium lauryl sulfate (SLS). All chemicals except Pt were evaluated in LLNA by the other authors, and the EC3 values were published by these authors (Basketter et al. 1996, 1998, 2003, 2004, 2007; Kimber et al. 1998; Ryan et al. 2000, 2002; Gerberick et al. 2004). All tested chemicals except LI were purchased from Sigma-Aldrich (St. Louis, MO, USA). LI was purchased from Wako Pure Chemicals (Osaka, Japan). The 14 water-

soluble chemicals (PD, GA, HQ, PPD, FA, ED, Ni, Py, Pt, Rc, BZC, LA, GC, and SLS) were first solubilized in saline, while water-insoluble chemicals (p-BQ, 1-BA, DNCB, PG, IE, CA, PAA, 2-MBT, HCA, EU, LI, AA, MP, VA, and 4-HBA) were solubilized in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture media was always less than 0.2%.

Pre-study cytotoxicity test (PI assay)

THP-1 cells were cultured in 24-well plates (1.0×10^6 cells per 1 ml per well) or 96-well plates (1.6×10^5 cells per 160 μ l per well) with various concentrations of chemicals for 24 h. Cells were washed twice, then stained with propidium iodide (PI; 0.625 μ g/ml as the final concentration), and cell viability was measured by using flow cytometry. Total events for living cell counting were 10,000 for 24-well plate method or 5,000 for 96-well plate method.

For water-soluble chemicals, the concentration that inhibited cell viability to 50% (IC₅₀) was calculated from the cell viability data. Based on the value of IC₅₀, we selected more than 10 doses which covered the range of concentrations from strong cytotoxicity (cell viability <30%) to almost no cytotoxicity (cell viability >95%). The ratio of serial dilution was changed according to the slope of the survival curve of each chemical (i.e., 1.2 to 1.8). If the IC₅₀ could not be determined with a concentration up to 5,000 μ g/ml, then the maximum dose for that test chemical was set at 10,000 μ g/ml (the highest technical dose; HTD) using a serial dilution of 1.5.

For water-insoluble chemicals, 12 concentrations causing 95% to 40% cell viability at 5% intervals and a 13th concentration causing 20% cell viability were calculated from the cell viability data. For instance, the concentration that produced 95%, 85%, 75%, 65%, and 50% cell viability showed CV₉₅, CV₈₅, CV₇₅, CV₆₅, and CV₅₀ (same as 50% inhibitory concentration; IC₅₀), respectively. If a chemical did not have any cytotoxicity at the HTD, eight doses were prepared using a serial dilution of 1.3 of the HTD. The HTD was set at 1,000 μ g/ml for the water-insoluble chemicals due to the maximum solubilization of a chemical in DMSO and the maximum dose of DMSO in cell culture medium. The cell viability determined in this cytotoxicity assay was termed the "Estimated Cell Viability". Cytotoxicity was also measured in conjunction with measurement of the relative fluorescence intensity

(RFI). The cell viability determined from these measurements was termed the “Actual Cell Viability” and was used to compare to the “Estimated Cell Viability” or CV95, CV85, CV75, etc. See section entitled “Flow cytometric analysis and mAbs” for method.

Being the first studies for this method, measurement of the estimated cell viability varied slightly between the water-soluble and water-insoluble chemicals. Both protocols were used in this study to determine if one approach was better than the other.

Chemical treatment of THP-1

THP-1 cells were plated at 1×10^6 cells/ml and treated for 24 h with each chemical. The test concentrations of each test chemical are listed in Tables 1 and 2. Ten test concentrations of each chemical were prepared to obtain a strong cytotoxicity response (cell viability <20% or 30% depending on chemical solubility) to almost no cytotoxicity (cell viability >95%) based on the result of pre-study cytotoxicity test (estimated cell viability). For water-soluble chemicals, a serial dilution was applied based on the IC50. A serial dilution of 1.2, 1.3, 1.5, or 1.8 was selected for each chemical according to the slope of the THP-1 cell survival curve. For water-

insoluble chemicals, CV95 to CV40 at five intervals and CV20, a total of 13 concentrations, were prepared.

Flow cytometric analysis and mAbs

After treatment with chemicals for 24 h, Fc receptor blocking procedure was conducted: 0.01% of Globlins Cohn fraction II and III (Sigma-Aldrich) were added for 10 min on 4°C. The fluorescence intensities of the THP-1 cell surface markers were then analyzed by flow cytometry (FACS Calibur Cell Quest, Becton Dickinson, San Jose, CA, USA). Cell staining was performed using the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark), anti-human CD86 (clone; Fun-1) from BD-PharMingen (San Diego, CA, USA); FITC-labeled mouse IgG1 (clone; DAK-G01) from DAKO. Using the manufacturer's recommended dilutions, cells were incubated with the above mAbs at $6 \mu\text{l}/3 \times 10^5$ cells/50 μl for the anti-human CD86 mAb and $3 \mu\text{l}/3 \times 10^5$ cells/50 μl for the anti-human CD54 mAb. In addition, FITC-labeled mouse IgG1 as an isotype control was used at a dilution of $3 \mu\text{l}/3 \times 10^5$ cells/50 μl . Cells were incubated with these mAbs for 30 min at 4°C. After

Table 1 List of water-soluble chemicals tested and test concentrations used

Chemical	Test concentration ($\mu\text{g}/\text{ml}$)		
	IC50	Highest dose	Serial dilution rate
Allergens (10)			
Potassium dichromate (PD)	8.6	60	1.5
Glutaraldehyde (GA)	7.6	25	1.3
Hydroquinone (HQ)	85.4	500	1.8
<i>p</i> -Phenylenediamine (PPD)	104	300	1.5
Formaldehyde (FA)	22.5	34.1	1.3
Ethylene diamine (ED)	797	1,000	1.5
Nickel sulfate (Ni)	790	2,000	1.5
Resorcinol (RC)	1,458	5,000	1.5
Pyridine (Py)	>5,000	10,000	1.5
Ammonium tetrachloroplatinate (Pt)	130	267	1.5
Non-allergens (4)			
Benzalkonium chloride (BKCl)	1.4	4.3	1.2
Glycerol (GC)	>5,000	10,000	1.5
Lactic acid (LA)	>5,000	10,000	1.5
Sodium lauryl sulfate (SLS)	71.2	100	1.2

THP-1 cells were treated with more than 10 doses for each of the 14 water-soluble chemicals tested. The highest dose was based on the cytotoxicity of each water-soluble chemical. A serial dilution rate based on IC50 was used except for pyridine, glycerol, and lactic acid. The dilution rate depended on test chemicals (1.2–1.8). Pyridine, glycerol, and lactic acid did not have any cytotoxicity and were evaluated using a serial dilution of 1.5 from 1,000 $\mu\text{g}/\text{ml}$ as the highest technical dose.

Table 2 List of water-insoluble chemicals tested and test concentrations used

Chemical	Test concentration (µg/ml)		
	CV50(IC50)	CV95	CV20
Allergens (11)			
<i>p</i> -Benzoquinone (p-BQ)	8.6	2.5	14.1
1-Benzoylacetone (1-BA)	162.8	8.4	336.7
Dinitrochlorobenzene (DNCB)	7.7	1.9	11.2
Propyl gallate (PG)	259.0	11.3	500.0
Isoeugenol (IE)	168.5	42.5	220.3
2-Mercaptobenzothiazole (2-MBT)	159.9	34.6	221.6
Cinnamic aldehyde (CA)	71.4	9.3	125.0
Phenylacetaldehyde (PAA)	39.8	4.9	56.6
Hexylcinnamaldehyde (HCA)	42.7	10.0	50.8
Eugenol (EU)	213.8	36.5	361.9
Lilial (LI)	47.0	17.70	67.4
Non-allergens (4)			
Acetanisol (AA)	508.3	70.30	808.30
4-Hydroxybenzoic acid (4-HBA)	*1		
Methylparaben (MP)	608.4	88.40	914.40
Vanillin (VA)	ND (>1000)	149.20	*2

The table lists only three concentrations for each chemical tested: CV50 (IC50), CV95, and CV20. However, THP-1 cells were treated with 13 doses based on the cytotoxicity of 13 water-insoluble chemicals, except for 4-hydroxybenzoic acid (4-HBA) and vanillin (VA). Twelve concentrations causing 95% to 40% cell viability at 5% intervals and a 13th concentration causing 20% cell viability were calculated from concentrations that were estimated to produce 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, and 40% cell viability, respectively, for each chemical except 4-HBA and vanillin. For 4-HBA, eight concentrations were tested since no cytotoxicity was observed and concentrations were based on a serial dilution of 1.3 from the highest concentration tested (1,000 µg/ml). For VA, eight doses (CV95, CV90, CV85, CV80, CV75, CV70, CV65, and CV60) were used, as the maximum concentration could only produce a CV60. *1 No cytotoxicity observed so eight doses were used and were based on serial dilution of 1.3 from 1,000 µg/ml; *2 the max dose of 1,000 µg/ml gave a 60% cell viability. For VA, eight doses (CV95, CV90, CV85, CV80, CV75, CV70, CV65, and CV60) were used.

washing and resuspending with phosphate-buffered saline supplemented with 0.1% bovine serum albumin, flow cytometry analysis was performed using a flow cytometer. PI solution was used at a concentration of

0.625 µg/ml after which dead cells were gated out. A total of 10,000 living cells were analyzed. RFI was used as an indicator of CD86 and CD54 expression and was calculated by the following formula:

$$\text{RFI}(\%) = \frac{\text{MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype control cells}}{\text{MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells}} \times 100$$

MFI = (Geometric)Mean fluorescence intensity

Experiment acceptability criteria and threshold for positive response

Three independent experiments were done for all concentrations, and mean RFI and cell viability [mean and standard deviation (SD)] values were calculated from these three independent experiments. The mean

RFI value was used to determine if concentrations gave a positive or negative response (i.e., enhancement of surface marker expression or not). Statistical analyses of the RFI values were not conducted. A response was considered to be positive when the mean RFI values for all three experiments and the RFI values of at least two of the three experiments

exceeded the threshold selected. A test concentration with an RFI value above the threshold selected for only one of the three experiments was not acceptable as a positive response. Cell viability (actual cell viability) was determined by PI assay. If actual cell viability for even one independent experiment was less than 50%, RFI of this tested concentration was not calculated and included in the evaluation because of diffuse labeling cytoplasmic structures due to cell membrane destruction (Becker et al. 1994).

Using the mean RFI values for each allergen, we calculated the estimated minimum concentration required to induce a threshold positive response: RFI=150 for CD86 and RFI=200 for CD54. The former was the estimated concentration of 150 for CD86 [EC150 (CD86)] and the latter was the estimated concentration of 200 for CD54 [EC200(CD54)].

Statistical analysis

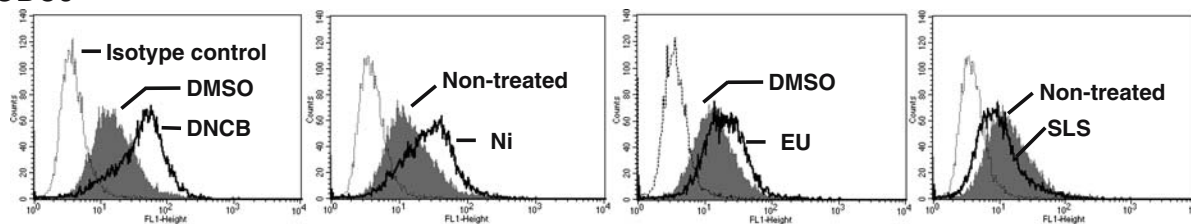
The Pearson test was used to establish a statistical significance between RFI values and cell viabilities and threshold values of LLNA and h-CLAT. *P* value less than or equal to 0.01 was considered statistically significant.

Results

Histogram of CD86/CD54 expression and MFI/RFI values

MFI values for a total of 29 chemicals were obtained by flow cytometric analysis. In Fig. 1, representative histograms of CD86/CD54 expression are shown for a strong response (induced by DNCB and Ni), weak response (as represented by EU), and a non-response (as indicated with SLS). MFI for cells treated with DNCB and Ni moved to high intensity compared with MFI for DMSO-treated cells or non-treated cells. For comparison, an example of the raw data obtained, which was obtained on a different day/laboratory as data displayed in histogram, for one independent experiment for DNCB and SLS are shown in Table 3. RFI value of both CD86 and CD54 for cells treated with DNCB and SLS were calculated from MFI values using the formula indicated in “Materials and methods”. The RFI values for cells treated with DNCB, as representative for the allergens, were over a threshold set for either CD86 or CD54, where test concentrations caused cell viability from 82.1% to 92.2%. On the other hand, the RFI values for cells

CD86



CD54

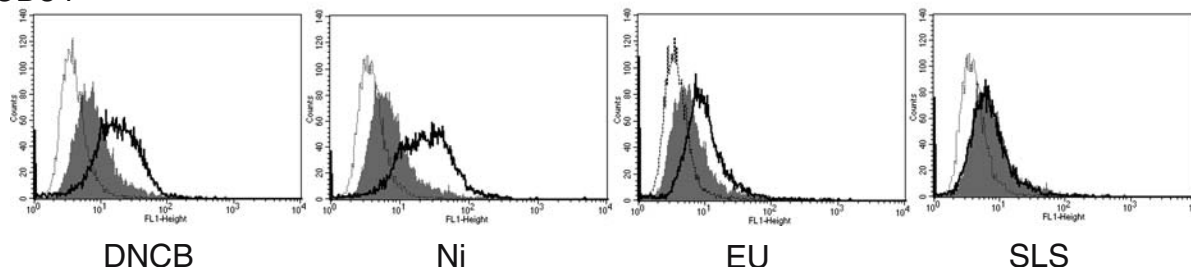


Fig. 1 Histogram of CD86/CD54 expression after DNCB, Ni, EU, or SLS treatments. Alteration of CD86 and CD54 expression induced by DMSO (vehicle), DNCB (2.5 $\mu\text{g/ml}$), NiSO₄ (85 $\mu\text{g/ml}$), EU (54 $\mu\text{g/ml}$), and SLS (58 $\mu\text{g/ml}$). Isotype control (dotted line), vehicle-control (either DMSO or

untreated; *shaded peak*), and chemical-treated (*dark solid line*). Because untreated control and DMSO-treated control gave the same results, untreated and DMSO-treated results were interchanged. These are representative data from three independent experiments. *MFI* (Geometric) Mean fluorescence intensity

Table 3 MFI and RFI values of DNCB and SLS treated experiments

Sample	Dose (µg/ml)	MFI			RFI		Cell viability (%)
		CD86	CD54	Isotype	CD86	CD54	
Control		13.6	6.9	3.4			96.0
DMSO	0.2%	14.7	7.2	3.4			95.9
DNCB	0.9	17.0	7.5	3.5	118.3	106.1	95.4
	1.3	18.3	7.3	3.5	129.8	100.0	95.5
	1.7	24.9	8.8	3.6	187.5	135.9	92.2
	2.0	36.6	10.6	3.7	290.1	183.6	88.5
	2.3	40.1	11.5	3.7	320.5	205.5	87.8
	2.8	49.6	15.4	3.5	405.5	313.5	83.4
	3.2	49.3	15.1	3.5	402.9	308.2	82.3
	3.4	46.9	14.3	3.4	382.9	289.7	83.3
	3.6	43.5	15.0	3.4	353.2	307.7	82.7
	3.8	43.3	14.3	3.3	351.5	288.7	82.1
Control		10.2	8.1	3.3			93.0
SLS	16.2	9.1	8.5	3.8	76.5	99.6	93.1
	19.4	8.5	8.6	3.7	69.5	102.5	92.3
	23.3	8.9	7.8	3.6	76.8	87.3	92.8
	27.9	9.4	7.3	3.5	87.0	81.7	92.6
	33.5	9.2	7.6	3.5	82.8	86.1	92.8
	40.2	10.0	7.3	3.3	97.1	82.7	94.2
	48.2	9.7	6.8	3.6	89.7	67.7	91.9
	57.9	8.4	8.1	3.7	68.7	91.8	90.1
	69.4	9.5	7.6	3.4	89.4	89.2	77.5
	83.3	8.1	7.7	3.7	65.3	84.6	54.2

The data in this table (MFI, RFI, and cell viability) were obtained from a couple of independent experiments for DNCB and SLS treatments. These experiments were evaluated on a different day and different laboratories. Ten concentrations were used for each chemical, and each MFI of each test dose was obtained by flow cytometry analysis. Using MFI values of isotype control and CD86/CD54, RFI values of CD86 or CD54 were calculated.

treated with SLS, the non-allergen representative, were not over the threshold set for both CD86 and CD54 at any dose although cell viability ranged from 54.2% to 93.1%.

Protocol selection for water-soluble and water-insoluble chemicals

As for the selection of protocols for water-soluble and water-insoluble chemicals, we could not decide which protocol was better before evaluating the relationship between CD86/CD54 expression and THP-1 cell viability. Therefore, we used two different protocols for setting up the concentrations for the water-soluble and the water-insoluble chemicals. However, with both protocols, we could evaluate each set of chemicals using more than 10 doses from non-toxic to toxic. Although the used protocols were different, we were able to evaluate the relationship between CD86/CD54 and cell

viability for both water-soluble and water-insoluble chemicals due to the larger number of doses tested.

Predicted performance for cell viability by PI assay for water-insoluble chemicals

Thirteen doses were used for each chemical except VA and 4-HBA. For VA, the HTD was CV60, so we evaluated eight doses from CV60 to CV95 with five intervals. 4-HBA showed no cytotoxicity at the HTD (1,000 µg/ml), so eight doses with a 1.3 serial dilution from 1,000 µg/ml were used. The test doses for the other water-insoluble chemicals were selected from the dose response curve obtained in the pre-study cytotoxicity test (PI assay): CV95 to CV40 with five intervals and CV20 that provided a 95% to 40% cell viability with 5% intervals and a 20% cell viability, respectively. The actual cell viability was obtained during the concurrent measurement of surface marker

expression in the definitive study as described in “Materials and methods.”

Table 4 shows the mean value of the actual cell viability and gives the percent of viable cells expected to be present (estimated cell viability) when exposed to the same concentration of chemical. When the cell viability for the estimated cell viability was compared to the actual cell viability, a good correlation in viability was observed with the higher concentrations (e.g., CV95 and CV80). In addition, the SD values and coefficient values of CV95, CV90, CV80, CV75, and CV70 were less than 10, which indicate that these doses showed a good reproducibility. Overall, there was a tendency that the higher cell viability gave better reproducible results. As such, the SD value and coefficient value were larger when cell viability was lower. A successful test (i.e., cell viability was over 50%) for each independent experiment at a concentration higher than CV65 (i.e., CV95, CV90, CV85, CV80, CV75, and CV70) was observed for all chemicals except HCA. For HCA, only CV75 at a concentration higher than CV65 had one independent experiment with less than a cell viability of 50% (data not shown).

The alteration of CD86/CD54 expression

The alteration of CD86 and CD54 expression after a 24-h treatment are calculated as an RFI value. The relationship between CD86/CD54 expression and cell viability for eight allergens (i.e., DNCB, PD, PG, FA, PAA, Ni, EU, and Py) and two non-allergens (i.e., MP and SLS) is shown in Figs. 2 and 3.

Each graph in Fig. 2 represents a chemical tested and reflects the changes to CD86 (dark square box line) and CD54 (open circle line) expression as well as cell

viability (open triangle broken line) at each concentration tested. The positive response pattern of CD86 and CD54 expression was different depending on allergen, which could be divided into mainly three groups. The first pattern caused by one set of chemicals was an augmentation of both CD86 and CD54. This chemical grouping consisted of DNCB, FA, PAA, Ni, EU, and Py. For PAA and Ni, CD86 expression reached about an RFI of 200, but CD54 expression was about four or seven times greater than CD86. For FA, only one concentration of more than 10 tested concentrations caused an increase in marker expression over RFI threshold (RFI=150 or 200). The second pattern in positive response caused by another set of chemicals was an augmentation only in CD86 expression. ED was a typical chemical of this grouping. The third pattern was an augmentation only in CD54 expression. PD and PG were involved in this latter pattern.

In addition to the patterns in response of surface marker expression, there are also mainly three patterns for the relationship between CD86/CD54 expression and cell viability. Figure 3 shows the cell viability and RFI value for each test dose from each independent test for four chemical representatives. The first pattern is an inverse, linear relationship. When cell viability was decreased, the RFI value of CD86/CD54 was increased. For example, expression of CD54 after treatment with PD and Ni, and expression of CD86 for EU has such a pattern. A good correlation between cell viability and marker expression was obtained for these responses ($P<0.01$). The second pattern was a bell-shape relationship where a maximum is obtained with a mid-level test concentration. Examples of this pattern are the results for expression of both CD86 and CD54 when cells are treated with DNCB or for expression of CD86 when cells are treated with Ni.

Table 4 Estimated cell viability and actual cell viability for water-insoluble chemicals except for 4-HBA

	CV95	CV90	CV85	CV80	CV75	CV70	CV65	CV60	CV55	CV50
Estimated cell viability (%)	95	90	85	80	75	70	65	60	55	50
Actual cell viability (%)										
Mean value	94.9	86.9	81.4	80.0	77.6	72.6	62.7	57.0	50.8	44.3
Standard deviation value	2.2	8	10.8	6.8	7.4	6.3	13.8	16.8	19.1	17.9
Coefficient of variation value	2.3	9.2	13.2	8.5	9.5	8.6	21.92	9.4	37.5	40.3

Cell viability determined before treatments with test chemicals (estimated cell viability) using the PI assay is compared to the cell viability determined concurrently with surface marker expression after treatment with test chemicals (actual cell viability). In both cases, cell viability was determined using flow cytometry analysis. The actual cell viability of each test concentration is shown as the mean, SD, and CV. As 4-HBA was not cytotoxic, the data for 4-HBA is not included in the table. VA is included only from CV95 to CV60.

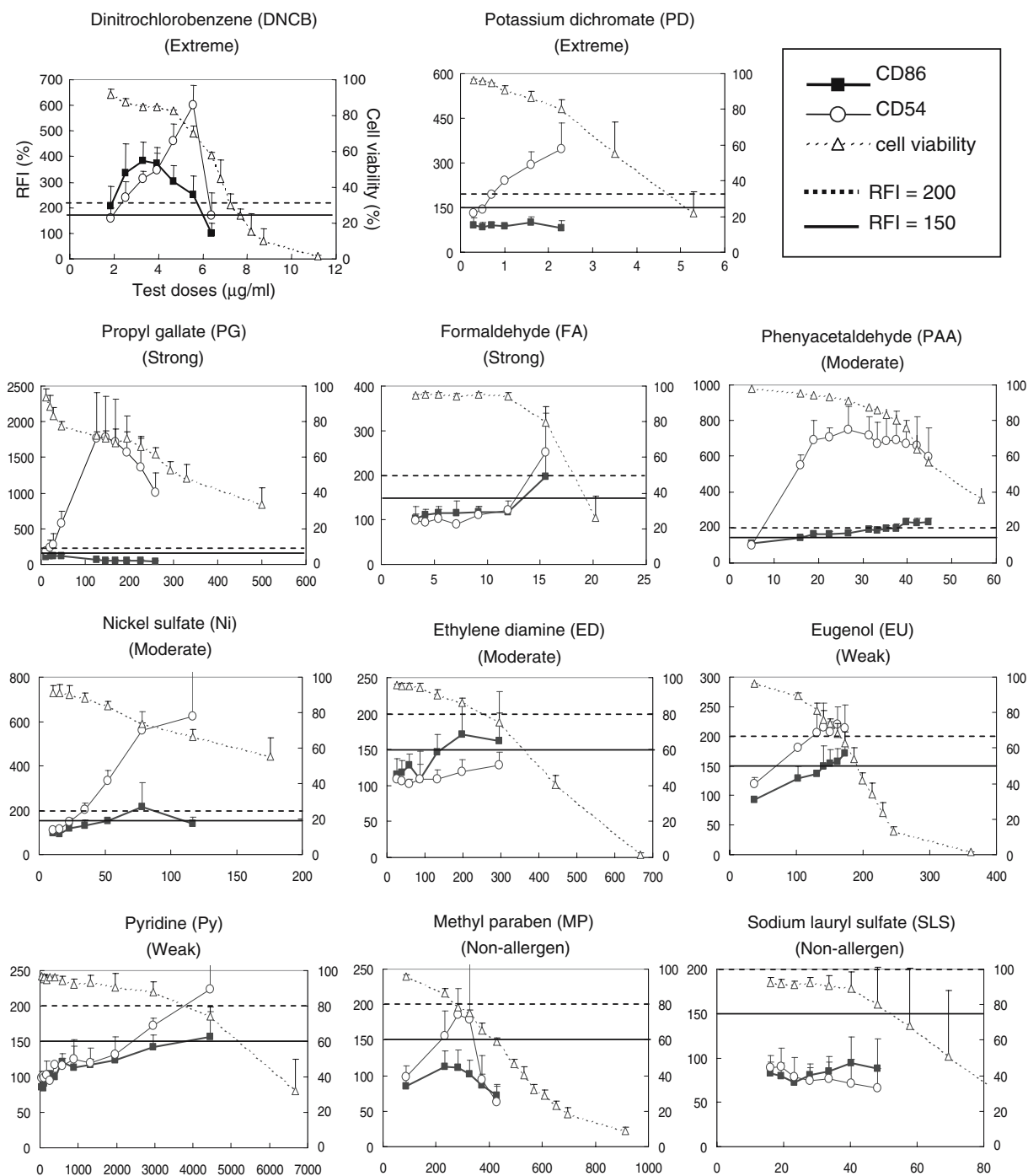


Fig. 2 Examples of the alteration of CD86/CD54. The alteration of CD86 (dark squares) and CD54 (open circles) expression and observed cell viability (open triangles) for 11 test chemicals is presented as a function of test concentration. The lines across the graph correspond to RFI values of 150

(solid line) or 200 (broken line). Solid line is criterion level for a positive response as indicated by augmentation of CD86 expression, and the broken line corresponds to the criterion level for CD54 expression

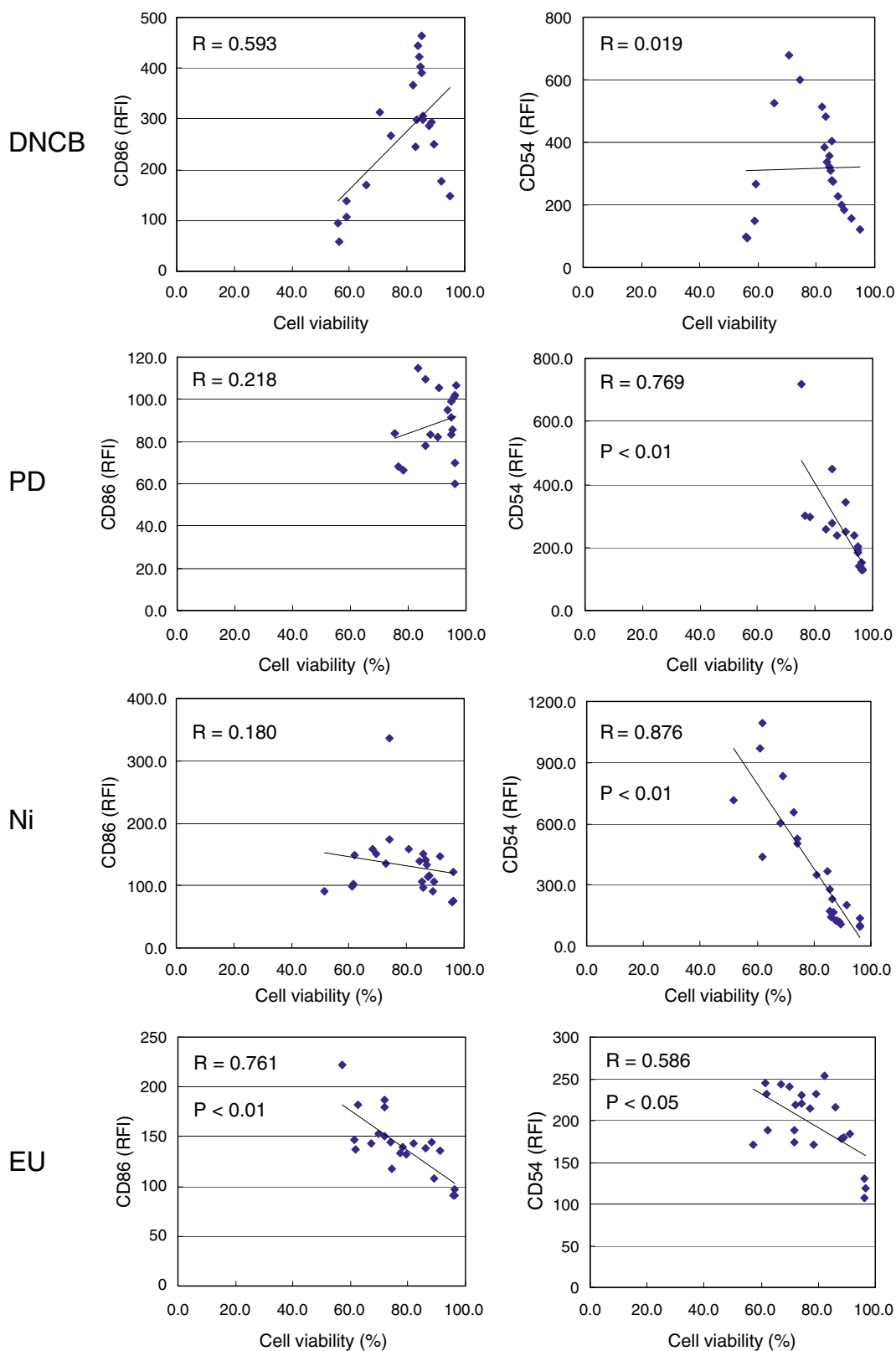


Fig. 3 Examples of the relationship between CD86/CD54 expression and cell viability. The relationship between of CD86 expression and observed cell viability (*left side*), and CD54 expression and observed cell viability (*right side*) for four test chemicals are presented. The individual results from each independent test for three experiments is provided (not mean data of three experiments). The fit regression was determined for each set of data, and a comparison between these values was completed using the Pearson test

The third pattern is a lack of relationship between CD86/CD54 expression and cell viability. In most of the cases where no relation is observed for the allergens, no expression of CD86 or CD54 was observed. An example of this pattern is the result of the expression of CD86 after treatment with PD.

For non-allergens, the effect on CD86 and CD54 marker expression was significantly different. For SLS, CD86 and CD54 expression did not change. From the SLS data in Fig. 2, an acceptable cell viability score was 80.2%, as the next higher dose had one experiment that gave less than 50% cell viability (below our acceptable criteria). Results from the independent tests gave RFI values for CD86/CD54 expression of 65.3/84.5, 52.9/42.6, and 89.7/89.2 for cell viability of 54.2%, 56.2%, and 67.0%, respectively (data not shown). These data show that SLS is irritant with strong cytotoxicity, but SLS did not induced augmentation of CD86/CD54 expression at any dose tested. However, MP caused a dose-related alteration in CD54 expression (RFI did not exceed 200) but not in CD86 expression.

Accuracy

The threshold for classifying a chemical as either an allergen or non-allergen was defined as an RFI value of 150 or 200 for CD86 and CD54 expression. Using these criteria, we evaluated 29 test chemicals (Table 5). For CD86 expression, 14 of 21 chemicals, known allergens, met the criterion of an RFI of 150 or more and were considered to be allergens, while eight non-allergens did not have an RFI above 150 and were considered to be non-allergens. The h-CLAT correctly identified these 22 chemicals. Based on the criterion for CD86 expression, 1-BA, PD, PG, IE, 2-MBT, HCA, and LI were not predicted correctly. When we set the criterion for classification up to an RFI of 200 or more, the non-allergens were found to be correctly identified as non-allergens, but only 8 of 21 known

allergens (DNCB, GA, HQ, PPD, CA, PAA, Ni, and Pt) were identified as allergens. From these data, the criterion of an $\text{RFI} \geq 150$ for CD86 expression gave a 75.9% accuracy and correctly identified more chemicals than a criterion of $\text{RFI} \geq 200$ for CD86.

For CD54 expression, when the criterion was an RFI of 150 or more, 20 of 21 known allergens met the criterion of an RFI of 150 or more and were considered to be allergens. Only one chemical, ED, was a false negative when this criterion was used. For known non-allergens, three of eight chemicals also met the criterion for being an allergen and would be considered to be allergens under the test conditions. The three chemicals, AA, BKC, and MP, were false positives when using this criterion. When the criterion was changed to an RFI of 200 or more, 17 of 21 known allergens were correctly identified as allergens, but four chemicals, HQ, IE, ED, and HCA, were false negatives when using this criterion. Eight non-allergens were correctly identified as non-allergen. From these data, the accuracy did not differ with either RFI criteria: $\text{RFI} \geq 150$ or $\text{RFI} \geq 200$. However, the criterion of an $\text{RFI} \geq 150$ for CD54 expression is a better criterion than a criterion of an $\text{RFI} \geq 200$, as fewer false negatives were observed. On the other hand, the criterion of an $\text{RFI} \geq 200$ is a better criterion than a criterion of an $\text{RFI} \geq 150$, as fewer false positives were observed. When we compared between CD86 and CD54, the accuracy of CD54 expression criteria is higher than the accuracy obtained with CD86 criteria.

In Table 6, a comparison of the in vivo (mainly LLNA) data to the h-CLAT data is conducted. We calculated sensitivity (%), specificity (%), positive predictivity (%), negative predictivity (%), and accuracy (%) for “CD86” and “CD54” and combinations of both (i.e., battery marker) using the criterion of $\text{RFI} \geq 150$ or $\text{RFI} \geq 200$ for each marker or combination. For sensitivity (%), CD54 was a better marker than CD86, and both “ $\text{CD54} \geq 150$ ” and “ $\text{CD86} \geq 200$ or $\text{CD54} \geq 150$ ” gave the highest sensitivity at 95.2%. For specificity (%), the criteria that correctly identified the non-allergens using the h-CLAT also gave 100% specificity: These were the criteria of “ $\text{CD86} \geq 150$,” “ $\text{CD86} \geq 200$,” and “ $\text{CD54} \geq 200$.” The criteria of “ $\text{CD86 or CD54} \geq 200$ ” and “ $\text{CD86} \geq 150$ or $\text{CD54} \geq 200$ ” also gave 100% specificity. In determining positive predictivity, the criteria that gave 100% specificity also gave 100% positive predictivity. For

Table 5 Alteration of CD86 and CD54 expression following exposure to twenty-nine chemicals

Chemical	LLNA*1		CD86		CD54	
	Potency	EC3 (%)	≥150	≥200	≥150	≥200
<i>p</i> -Benzoquinone (p-BQ)	Extreme (4)	0.0099	+	–	+	+
1-Benzoylacetone (1-BA)		0.04 *2	–	–	+	+
Dinitrochlorobenzene (DNCB)		0.05	+	+	+	+
Potassium dichromate (PD)	Strong (5)	0.08	–	–	+	+
Glutaraldehyde (GA)		0.1	+	+	+	+
Hydroquinone (HQ)		0.11	+	+	+	–
p-Phenylenediamine (PPD)		0.16	+	+	+	+
Propyl gallate (PG)		0.32 *2	–	–	+	+
Formaldehyde (FA)	Moderate (7)	0.61	+	–	+	+
Isoeugenol (IE)		1.2	–	–	+	–
2-Mercaptobenzothiazole (2-MBT)		1.7	–	–	+	+
Ethylene diamine (ED)		2.2 *3	+	–	–	–
Cinnamic aldehyde (CA)		3.0	+	+	+	+
Phenylacetaldehyde (PAA)		3.0	+	+	+	+
Nickel sulfate (Ni)		4.8 *4	+	+	+	+
Resorcinol (RC)		5.7 *5	+	–	+	+
Hexylcinnamaldehyde (HCA)	Weak (4)	11	–	–	+	–
Eugenol (EU)		13	+	–	+	+
Lilial (LI)		19	–	–	+	+
Pyridine (Py)		72	+	–	+	+
Ammonium tetrachloroplatinate (Pt)	No data	No data	+	+	+	+
Acetanisoole (AA)	Non sensitizer (7)		–	–	+	–
Benzalkonium chloride (BKC)		*6	–	–	+	–
Glycerol (GC)		*7	–	–	–	–
4-Hydroxybenzoic acid (4-HBA)			–	–	–	–
Lactic acid (LA)		–	–	–	–	–
Methylparaben (MP)		*8	–	–	+	–
Vanillin (VA)		*9	–	–	–	–
Sodium lauryl sulfate (SLS)	false positive	*7	–	–	–	–

The extent of augmentation of CD86 and CD54 expression is listed for the 29 chemicals tested. The results for each chemical are given for two criterion levels for each surface marker. A positive sign (+) indicates that one or more test concentration of the chemical augmented marker expression above the set criterion: RFI≥150 or RFI≥200. A negative sign (–) indicates that there was no test concentration that augmented marker expression above the criterion. Also presented in the table are the reported EC3 values from a LLNA and chemicals are grouped according to their reported potency. *1 Gerberick et al. 2004; *2 Basketter et al. 2004; *3 Kimber et al. 1998; *4 Ryan et al. 2002; *5 Basketter et al. 2007; *6 Basketter et al. 1998; *7 Basketter et al. 1996; *8 Ryan et al. 2000; *9 Basketter et al. 2003

negative predictivity, only the criteria of “CD86 or CD54≥150” gave 100%. The criteria of “CD86≥150 or CD54≥200” gave the highest accuracy at 93.1%. Based on these findings, we set the criteria of “CD86≥150 or CD54≥200” as the current tentative criteria to be used in this study.

Cell viability and dose range of positive response

The cell viability range and test concentrations that gave a positive response (CD86≥150 and CD54≥200) for 21 allergens are shown in Table 7.

For the marker CD86, 14 known allergens were positive and 7 known allergens, 1-BA, PD, PG, IE, 2-MBT, HCA, and LI, did not meet the criteria for a positive response. As the latter represents a negative response, no cell viability or dose are listed in Table 6. Some allergens (FA, CA, Ni, and Py) had a positive response only at one tested concentration when the criterion of CD86≥150 was used. This indicates that some allergens are positive or have a positive response only a narrow cell viability and concentration range. On the other hand, HQ and PAA were positive over a very wide range of cell viability, 58% to 90% and 56%

Table 6 Effectiveness of surface marker criterion thresholds

Marker	Criteria of RFI	Sensitivity (%)	Specificity (%)	Positive predictivity (%)	Negative predictivity (%)	Accuracy (%)
CD86	≥150	66.7	100	100	53.3	75.9
	≥200	38.1	100	100	38.1	55.2
CD54	≥150	95.2	62.5	87.0	83.3	86.2
	≥200	81.0	100	100	66.7	86.2
Battery	CD86 or CD54≥150	100	62.5	87.5	100	89.7
	CD86 or CD54≥200	85.7	100	100	72.7	89.7
	CD86≥150 or CD54≥200	90.5	100	100	80.0	93.1
	CD86≥200 or CD54≥150	95.2	62.5	87.0	83.3	86.2

Each surface marker criterion threshold (alone or in combination) was evaluated for the sensitivity, specificity, positive predictivity, negative predictivity, and accuracy of the h-CLAT data to the known in vivo data (LLNA and guinea pig). Overall, the use of two markers with two different criterion thresholds gave the highest accuracy: CD86 marker with a criterion of RFI≥150 or CD54 marker with a criterion of RFI≥200 had the highest accuracy, 93.1%

Table 7 Cell viability and positive response test concentration ranges for 21 tested allergens

Test chemicals	Potency category	CD86≥150			CD54≥200		
		Range of cell viability (%)	Range of dose (μg/ml)	Ratio (highest/lowest dose)	Range of cell viability (%)	Range of dose (μg/ml)	Ratio (highest/lowest dose)
<i>p</i> -Benzoquinone	Extreme (4)	76–89	4.2–6.0	1.4	68–76	60–6.6	1.1
1-Benzoylacetone		–	–	–	55–92	51–163	3.2
Dinitrochlorobenzene		70–92	1.9–5.6	2.9	70–87	2.5–5.6	2.2
Potassium dichromate		–	–	–	80–90	0.7–2.3	3.2
Glutaraldehyde	Strong (5)	77–87	6.7–8.8	1.3	77	8.8	–
Hydroquinone		58–90	1.4–47.6	34	–	–	–
<i>p</i> -Phenylenediamine		59–82	5.2–88.9	17.1	59–64	26.3–88.9	3.4
Propyl gallate		–	–	–	62–77	45.7–259.0	5.7
Formaldehyde	Moderate (7)	80	15.5	–	80	15.5	–
Isoeugenol		–	–	–	–	–	–
2-Mercaptobenzothiazole		–	–	–	61–92	68.6–151.5	2.2
Ethylene diamine		75–86	197.5–296.3	1.5	–	–	–
Cinnamic aldehyde	Weak (4)	71	16.8	–	71	16.8	–
Phenylacetaldehyde		56–94	18.9–44.7	2.4	56–96	15.9–44.7	2.8
Nickel sulfate		74	78	–	66–88	34.7–117.1	3.4
Resorcinol		89–92	38.5–57.8	1.5	68.80	195.1–658.4	3.4
Hexylcinnamaldehyde	No data	–	–	–	–	–	–
Eugenol		63–74	150.7–173.3	1.2	63–81	131.0–173.3	1.3
Lilial		–	–	–	55	47	–
Pyridine		74	4444.4	–	74	4444.4	–
Ammonium tetrachloroplatinate		75–90	104–52.7	5	64–89	6.9–52.7	7.6

The cell viability range and test concentration range are provided for the 21 chemicals that gave a positive response. Some test chemicals had a positive response at only one dose, which did not allow for calculation of the ratio of highest to lowest dose with positive response. – No positive response was obtained for these test chemicals.

to 94%, respectively. Fourteen allergens were positive at a dose concentration that gave about 70% to 90% cell viability. For HQ and PPD, a positive response was observed in the wide-tested concentration range, 1.4–47.6 and 5.2–88.9 $\mu\text{g/ml}$, respectively. We calculated the ratio of highest tested concentration that gave a positive response to the lowest tested concentration that gave a positive response to better understand the dose response or slope of effect. For some allergens, p-BQ, GA, ED, RC, and EU, the ratio of highest to lowest dose tested concentration were 1.2 to 1.5. EU had a ratio of 1.2, the narrowest range, which shows that a positive response, was observed in a narrow concentration range.

For the marker CD54, 17 known allergens were positive, while HQ, IE, ED, and HCA had a negative response or were not predicted to be allergens. IE and HCA, two known allergens, were not predicted to be allergens by both CD86 and CD54. GA, FA, CA, and Py had a positive response only at one tested concentration when the criterion for CD54 of $\text{RFI} > 200$ was used. Both CD86 and CD54 markers found FA and Py to be positive only at one tested concentration. 1-BA and PAA were positive in the cell viability range of 55% to 92% and 56% to 96%, respectively. Seventeen allergens were positive at the dose that gave about 65% to 85% cell viability. The tendency for CD54 expression to be augmented at a dose that gave a lower cell viability than CD86 was observed. p-BQ and EU had a positive response in a very narrow test concentration range. The ratio of the highest tested concentration to give a positive response to the lowest tested concentration to give a positive response for p-BQ and EU were 1.1 and 1.3, respectively.

From these data, we demonstrated again that about 65% to 90% cell viability was needed to enhance

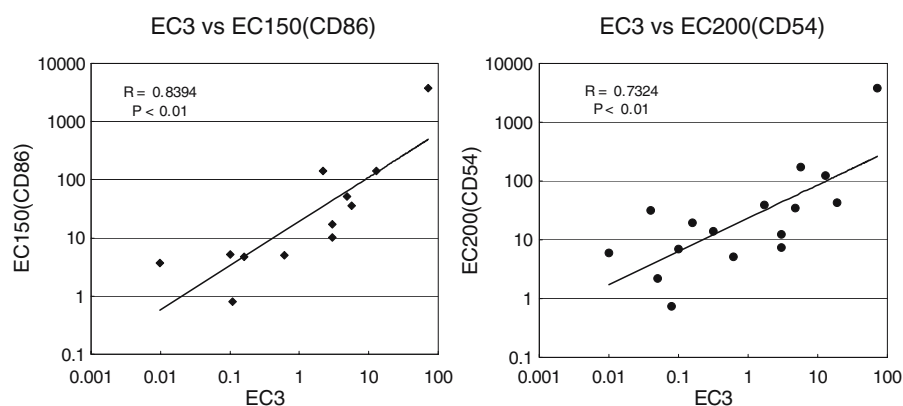
CD86 and/or CD54 expression. In addition, we found that some chemicals were only positive at a very narrow dose range. Therefore, the use of small serial dilution ratios and several test concentrations are needed to predict an allergen correctly.

The relationship between the LLNA EC3 and the h-CLAT EC150 (CD86) and EC200 (CD54)

We calculated the EC150(CD86) and EC200(CD54) for some allergens that caused an augmentation of CD86 and CD54 expression over threshold. For EC150 (CD86), there were 12 of 21 known allergens that caused an augmentation of CD86 expression. The 12 chemicals and EC150 values (in parenthesis) were p-BQ (3.7), GA (5.2), HQ (0.8), PPD (4.8), FA (5.2), ED (141.9), CA (10.3), PAA (17.2), Ni (52.2), RC (35.9), EU (142.5), and Py (3791.7). The EC200(CD54) was calculated for 16 of 21 allergens that caused an augmentation of CD54 expression, and these values were p-BQ (5.9), 1-BA (31.3), DNCB (2.2), PD (0.7), GA (6.9), PPD (19.3), PG (13.8), FA (38.1), 2-MBT (39.3), CA (12.4), PAA (7.3), Ni (34.4), RC (172.1), EU (124.2), LI (42.9), and Py (3751.3), respectively. The calculated EC3 values from the LLNA were plotted against the calculated EC150(CD86) and EC200(CD54) values from the h-CLAT in Fig. 4.

When we compared the h-CLAT EC150(CD86) or EC200(CD54) to the LLNA EC3 values for 12 or 16 chemicals, the chemicals with a larger EC150(CD86) and EC200(CD54) values had a tendency to have a bigger LLNA EC3 value. In Fig. 4, a good correlation is observed between LLNA EC3 value and EC150 (CD86) or EC200(CD54). The correlation coefficient was $r=0.8394$ ($P<0.01$) between EC3 and EC150

Fig. 4 Correlation of h-CLAT and LLNA thresholds. The correlation between the EC3 value of LLNA and the EC150 value for CD86 or EC200 value for CD54 from the h-CLAT is evaluated. The fit regression was determined for each set of data and a comparison between these values was completed using the Pearson test



(CD86). A lower correlation coefficient ($r=0.7324$, $P<0.01$) was obtained between the LLNA EC3 and EC200(CD54). Overall, the h-CLAT values significantly correlated with the LLNA EC3 values ($P<0.01$), but there was a better correlation between EC150 (CD86) and LLNA EC3.

Discussion

To distinguish between allergens and non-allergens correctly using the h-CLAT, we needed to evaluate appropriate test doses. Because some cytotoxicity occurred alongside the augmentation of CD86/CD54 expression, the dose-finding study becomes important in determining the appropriate test dose for the study. Cell viability was measured using flow cytometry with PI before starting the study and concurrently with cell surface marker expression. The PI assay before the start of the study was carried out to estimate the concentration at which cell viability could be estimated during the definitive study. In our previous study, Sakaguchi et al. (2007) reported the usefulness of the PI assay to decide appropriate test doses. In this study, a high reproducibility of mean cell viability was similarly confirmed for 14 water-insoluble test chemicals. A high reproducibility and low coefficient of variation values were obtained for test concentrations that estimated cell viability of 70% or more, except for the estimated 85% cell viability. The PI method was selected as the dose range finding method for the h-CLAT due to its good reproducibility and rapid measurement. For example, the Alamar Blue proliferation test measured Alamar Blue fluorescence 4 h after adding Alamar Blue (Azam et al., 2006). Compared to 4 h, the PI method is quicker at 15 min and better for the h-CLAT.

Basically, most allergens showed a dose-dependent alteration of CD86 and/or CD54 expression. But depending on chemical, the alteration pattern was different. Three patterns for positive response were observed with these test chemicals. The first pattern was an augmentation of both CD86 and CD54 markers after cell treatment with our test chemicals. The second set was augmentation of only CD86 expression, while the third pattern was augmentation of only CD54 expression.

In the induction phase of skin sensitization, both CD86 and CD54 as co-stimulatory molecules on

Langerhans cells have the important role of presenting antigen in vivo. Thus, we estimated that both markers would be up-regulated in the presence of allergens. Previously, we evaluated the effect of chemical treatment time (6, 12, 24, 48, or 72 h) on CD86 expression using DNCB and found that CD86 expression began to plateau at 24 h (Ashikaga et al. 2006). Then, we compared 24- and 48-h treatment times using nine chemicals, and the 24-h treatment showed a better accuracy (Sakaguchi et al. 2006). Therefore, we used 24 h as the treatment time in h-CLAT. Python et al. (2007) found that some allergens had time- and chemical-dependent effects on CD86 expression on U937 cells. 2,4,6-Trinitrobenzenesulfonic acid, *p*-toluylenediamine, and 4-amino-2-hydroxytoluene exhibited relevant exposure time-dependent increases in CD86 expression, while 2-amino-5-methylphenol had time-dependent decreases on CD86 expression. Earlier, Miyazawa et al. (2008) mentioned that the signaling pathway mechanism for CD54 expression on THP-1 cells were different between DNCB and NiSO₄ and resulted in different kinetics for these chemicals. From these data, augmentation of CD86 or CD54 expression appears to be test chemical and treatment time specific. These findings also suggest that the use of two markers of sensitization would provide an in vitro skin method that would better predict allergens. Python et al. (2007) also found that a combination of at least two markers was needed to establish a reliable evaluation of the DC activation potential.

From the fact that known allergens increased RFI values while non-allergens did not consistently increase RFI values, the use of RFI to distinguish between allergens and non-allergens seems appropriate. In our previous report, we set two tentative criteria: $\text{RFI} \geq 150$ or ≥ 200 (Sakaguchi et al. 2006). We found an accuracy of 100% when we used $\text{RFI} \geq 150$ for CD86 and/or $\text{RFI} \geq 200$ for CD54 (Sakaguchi et al. 2007). In the current study, the criterion, $\text{RFI} \geq 150$ for CD86 and/or $\text{RFI} \geq 200$ for CD54, also showed the highest accuracy (93.1%). Specificity and positive predictivity were also 100%. When this criterion was used, IE and HCA had a false negative result.

The false negative findings for IE and HCA may be the result of a lack of metabolic activation and poor solubility, respectively. IE was reported to be a pro-hapten (Smith and Hotchkiss, 2001), where metabolic activation is needed for allergy induction. THP-1 cells may not have enough metabolic activity

to form the hapten. HCA did not have a good solubility in DMSO in our study. To predict allergy potential of HCA, an appropriate vehicle with good solubility would be needed. Python et al. (2007) also could not predict allergy potential of HCA using U937 cells. MUTZ-3 cells treated with HCA resulted in dose-dependent expression of CD86 and CD54 (Azam et al. 2006). Besides the solubility issue, the different responses observed with these cell lines might reflect the individual variation.

To distinguish between allergen and non-allergen, a higher marker expression threshold may be appropriate. Ovigne et al. (2005) found that CD86 expression of 120% or more, above control, using U937 cells was needed to obtain a “positive”. Hulet et al. (2005) used a similar threshold value for CD86 expression with human peripheral blood-derived dendritic cells. Our suggested criterion threshold values for CD86 and CD54 expression were also similar to those previously reported. As such, we would like to use these thresholds as final criterion in the h-CLAT.

As a result of our evaluation using more than 10 doses for 21 known allergens, the relationship between cell viability and applied test concentration to obtain a positive reaction is better understood. Most allergens had a positive response within the range of 65% to almost 90% cell viability, although the range was different depending on the chemical and marker expression. Hulet et al. (2005) reported that approximately 10–15% cytotoxicity was observed with the up-regulation of CD86 expression. Azam et al. (2006) indicated that some cell surface markers (e.g., CD86 and CD54) were up-regulated at concentrations that produced greater than 10% reduction in MUTZ-3 cell proliferation. Python et al. (2007) also reported that most tested chemicals induced an up-regulation of CD86 expression on U937 cells with cytotoxic concentrations (i.e., cell viability <85%). In using THP-1 cells, we earlier reported that some allergens (e.g., DNCB and NiSO₄) augmented CD40 and CD83 expression and markedly induced cytokine (e.g., TNF- α and IL-8) secretion at some cytotoxic dose (Miyazawa et al. 2007). Our findings found that some cytotoxicity occurred with the activation of dendritic cells. In addition, we have found that MAPK phosphorylation (e.g., p38 and ERK) was related with augmentations of CD86/CD54 expression, and cytotoxicity was also present (Miyazawa et al.

2008). In our current findings, cells exposed to PAA caused an induction of CD86 and CD54 expression at almost a non-toxic dose (94% and 96% cell viability, respectively, from two independent tests; data not shown). Of course, a stronger augmentation of CD86/CD54 expression was noted for PAA with higher doses, but a greater cytotoxicity was also observed. From these data, we currently believe that the cytotoxicity and augmentation of sensitization responses (marker expression and cytokines production) observed in our system are indirectly related. The cytotoxicity may be a by-product of the test concentration that is needed to enhance the sensitization responses. However, we still need to clarify in more detail the mechanism involved.

Appropriate test doses are needed to better predict allergens. In our study, some allergens were positive at a very narrow dose range. This observation suggests that a small test concentration range is important for identifying the positive response range. Thus, the serial dilution should be small to capture the narrow response. p-BQ and EU are good examples. The ratio of highest to lowest test concentration with a positive response for p-BQ was 1.4 and 1.1 for CD86 and CD54 expression, respectively. For EU, the ratio was 1.2 and 1.3, respectively. The data for 14 water-insoluble chemicals showed that most allergens had a positive result at CV75 (data not shown). From these data, we proposed a “serial 1.2 dilution rate based on CV75” as one test dose setting. As for the total number of test concentrations, the more concentrations used will result in a wider range of cell viability. However, if the relative ease of the method is considered, eight doses can be done and would be appropriate.

Earlier, we suggested the possibility of using EC150 (CD86) or EC200 (CD54) to classify skin sensitization potency (Sakaguchi et al. 2007). In this study, we compared the h-CLAT EC150 (CD86) or EC200 (CD54) to the EC3 values from the LLNA for 12 of 21 allergens for CD86 or 16 of 21 allergens for CD54, respectively (Fig. 4). Both the h-CLAT and the LLNA are very different assays, but both have the potential to evaluate the sensitization potential of a chemical. Although the LLNA does evaluate a couple of variables in the sensitization process (bioavailability or exposure, protein reactivity of hapten, and LC/DC activation), the h-CLAT is primarily focused on assessing the potential of a chemical to enhance LC/

DC activation. Given the same chemical, with its intrinsic reactivity, a comparison between the enhancement of cell marker expression in the h-CLAT and the EC3 data of LLNA is possible. A good correlation was observed among both values ($P < 0.01$), but a better correlation was observed with EC150(CD86) and EC3. Other researchers also suggested a similar approach to classifying skin sensitization potency. Azam et al. (2006) calculated “Effective Concentration,” which was defined as the extrapolated concentration of the tested compound at which the cells start to give a positive signal using MUTZ-3 cells. The authors reported that this dose was proportional to strength of each sensitizer. Ovigne et al. (2005) suggested the usefulness of EC120, the concentration at which CD86 reaches 120% for rank classification using U937 cells. The latter suggestion of using concentration at which LC/DC activation marker expression (CD86 and/or CD54) reaches a certain threshold does have some useful possibilities for determining allergen rankings in a cell-based assay. However, because not enough chemicals have been evaluated, it is necessary to evaluate more chemicals to ascertain the possibility of the rank classification in h-CLAT using EC150(CD86) and EC200(CD54) as the criteria. Furthermore, we need to also evaluate the role of other variables involved in sensitization (e.g., bioavailability and protein reactivity) in combination with our h-CLAT. In general, the strength of an allergen could be determined by several factors, for example, chemical structural alert, bioavailability, protein reactivity, DC maturation, and T-cell proliferation (Jowsey et al. 2006). We would like to consider the usefulness of our EC150(CD86) [or EC200(CD54)] and other factors such as LogKow (bioavailability) and peptide reactivity assay data (protein reactivity), especially for some chemicals that did not show good correlation between EC150(CD86) and EC3 [or EC200(CD54) vs EC3].

Based on our current findings, we think that the h-CLAT may be a useful and robust cell-based in vitro skin sensitization test for evaluating various chemicals. We need to further confirm the applicability of h-CLAT and to further discern the possibility of a rank classification using h-CLAT by evaluating more chemicals. It is hoped that the h-CLAT in conjunction with other tests (skin penetration assay, protein/peptide reactivity assay) will be used as part of a battery screen and that these methods will replace animal testing for skin sensitization in the future.

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