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In vitro detection of contact allergens: Development of an optimized protocol using human peripheral blood monocyte-derived dendritic cells

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ABSTRACT

Allergic contact dermatitis is a delayed T-cell mediated allergic response associated with relevant social and economic impacts. Animal experiments (e.g. the local lymph node assay) are still supplying most of the data used to assess the sensitization potential of new chemicals. However, the 7th amendment to the EU Cosmetic Directive will introduce a testing ban for cosmetic ingredients after 2013. *In vitro* alternative methods are thus being actively developed. Although promising results have been obtained with cell lines, their reduced functionality and inherent genomic instability led us to reinvestigate the use of peripheral blood monocyte-derived dendritic cells (PBMDs) for the establishment of a reliable *in vitro* sensitization test. To solve the issues associated with the use of primary cells, the culture and exposure conditions (cytokine concentrations, incubation time, readout, pooled vs. single donors and cytotoxicity) were re-assessed and optimized. Here we propose a stable and reproducible protocol based on PBMDs. This should allow a wider acceptance of PBMDs as a reliable test system for the detection of human skin sensitizers and the inclusion of this protocol in an integrated testing strategy.

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1. Introduction

Allergic contact dermatitis (ACD) is a type-IV allergy of the skin associated with significant social and economic impacts. It has a high frequency in the general population and thus represents a significant public health challenge (Schafer et al., 2001). It is the results of a delayed T-cell-mediated allergic reaction to skin proteins modified by exposure to low molecular weight reactive chemicals (haptens). The source of haptens, also referred to as sensitizers, can be very diverse, e.g. antibiotics, antimycotics, metal ions or cosmetic ingredients. Therefore a comprehensive risk assessment of new chemicals is crucial to identify potential ACD inducers and avoid unacceptable exposure level of involved workers or end users. Currently, animal experiments (e.g. the local lymph node assay (LLNA) or the guinea pig maximization test) were supplying most of the data needed for risk assessment and decision-making (Basketter and Scholes, 1992; Kimber et al., 1994, 2002; Dearman et al., 1999; Gerberick et al., 2000; Basketter et al., 2002). However, the 7th Amendment to the European Cosmetics Directive (Directive 76/768/EEC) will severely restrict the use of animal tests. Skin sensitization tests are considered as re-

peated dose toxicity assays, and the deadline for the corresponding animal test ban has been set to 2013. After this date, the cosmetic industry will face a marketing ban for new ingredients examined in animal experiments. The development of *in vitro* alternative test methods for skin sensitisation is thus required by the cosmetic industry and is also raising much interest in other industry sectors. To develop these alternative *in vitro* methods, an array of approaches is being investigated. These range from biophysical/*in silico* approaches to cell based systems. For cellular assays, a variety of cells known to be involved in the biology of ACD is being evaluated, i.e. keratinocytes, dendritic cells (DCs) and T-cells. DCs are known to play a central role during the induction of skin sensitization through their ability to uptake, process and present haptenated self-proteins to T-cells, and represent the most abundant antigen presenting cell population in the skin (Novak et al., 1999; Toebak et al., 2009). *In vitro* assay development has thus rapidly focused on available sources of DC-like cells including primary cells (cord or peripheral blood) or cell lines (e.g. U937, THP-1, Mutz-3, KP-1, HL-60, K562, reviewed in dos Santos et al. (2009)). Assays based on primary cells require specific cell sources that might represent a limiting factor, especially in the case of cord blood based assays. Donor to donor variability was also noted as a critical point (Aiba et al., 1997; Basketter and Maxwell, 2007) and many research groups directed their efforts towards the devel-

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opment of *in vitro* test systems using cell line. Cell line based assays were expected to overcome the main limitations of the primary cells. Being widely available through cell banks and considered as easy to cultivate, cell lines represented an obvious alternative. Most cell lines evaluated were of myeloid origin, mainly from hematopoietic neoplasms. However, the genome of these tumorigenic and transformed cells is severely altered and due to their intrinsic genomic instability, there may accumulate further genomic abnormalities over prolonged culture period. As a consequence some lost their typical homeostasis and have only limited functional properties (e.g. U937 decreased response to lipopolysaccharide (LPS)). Moreover it is known that an adequate usage of these lines is limited by cell passages due to their progressive loss of response to chemical stress (Sakaguchi et al., 2010). Nevertheless, in case of the U937 and THP-1 cell lines, promising assays could be developed by using carefully controlled culture conditions ensuring a relatively stable cell phenotype (Ade et al., 2006; Ashikaga et al., 2006; Sakaguchi et al., 2006; Python et al., 2007). The h-CLAT and MUSST test protocols based on THP-1 or U937 cells respectively are now under consideration for pre-validation by the European Centre for the Validation of Alternative Methods (ECVAM). Another cell line, Mutz-3, is being intensively investigated; it is relatively immature and can be forced to differentiate into a Langerhans-like phenotype (Azam et al., 2006; Kim et al., 2006; Larsson et al., 2006; Nelissen et al., 2009; Python et al., 2009; Ouwehand et al., 2010). However, Mutz-3 propagation requires complex culture procedures (e.g. co-culture), thereby reducing their perceived advantages and inducing unexpected intra- or inter-laboratories variability.

The difficulties associated with cell lines led us to reinvestigate the use of peripheral blood monocyte derived DCs as a reliable *in vitro* test system for detecting skin sensitizers. As cell culture and exposure conditions described in the literature were not always consistent a fundamental protocol review and optimization phase was initiated (Degwert et al., 1997; Reutter et al., 1997; Aeby et al., 2004; Staquet et al., 2004). This included the determination of the optimal cell isolation procedure (use of positive or negative depletion protocols), the use of monocytes obtained from single donors instead of pooled cells and the optimization of the culture (cytokine concentrations) and exposure conditions. The result is an improved and stable protocol with reduced donor to donor variability, reproducible results and a significant correlation with the LLNA results. This should allow a wider acceptance of monocyte derived DCs as cells of choice for detecting human skin sensitizers.

2. Materials and methods

2.1. Tested chemicals

Tested chemicals were dissolved either in dimethylsulfoxide (DMSO) or ethanol and diluted in RPMI 1640 medium without phenol red (R7509, Sigma, Germany). Final in-well concentration of DMSO was 0.1%. The sensitizer 2,4-dinitrochlorobenzene (DNCB) and the irritant sodium dodecyl sulphate (SDS) were used as positive or negative controls, respectively. Ascorbic acid, dimethylphtalate, 4-hydroxybenzoic acid and benzalkonium chloride were selected as examples of non-sensitizers. Glutaraldehyde was tested as a typical strong sensitizer, nickel sulfate as an example of a metal sensitizer. Cinnamic aldehyde was selected as an example of a moderate sensitizer and α -hexylcinnamic aldehyde, eugenol and hydroxycitronellal represented weak sensitizers. SDS and DMSO were purchased from Merck, Germany (Merck #1.12533 and #1.02950); Hydroxycitronellal was purchased from BASF (#50001174) all other test chemicals in reagent grade and the lipo-

polysaccharide (LPS; L5014) used as a functional control, were purchased from Sigma, Germany.

2.2. Culture medium

Cells were cultured in RPMI 1640 without phenol red (R7509, Sigma, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS Gold, A15-151, PAA, Austria), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin (P11-013, PAA, Austria).

2.3. Purification of human monocytes and differentiation into PBMDCs

Mononuclear cells were enriched from buffy coats of healthy donors by ficoll (Lymphocyte Separation Medium LSM 1077, PAA, Austria) gradient density centrifugation as described elsewhere (Degwert et al., 1997). Buffy coats were randomly and anonymously obtained as residual product from whole blood preservation production. CD14⁺/CD14⁺ monocytes were then isolated by either positive selection using anti-CD14-Ig coupled magnetic microbeads (MACS CD14 MicroBeads, human (130-050-201); Miltenyi Biotec, Germany) or negative depletion (MACS Monocyte Isolation Kit II, human (130-91-153); Miltenyi Biotec, Germany) according to the manufacturer's instruction. The isolated monocytes were stored at -80°C for a maximum period of 3 months. Before differentiation to PBMDCs, cells were thawed and their CD14 phenotype was measured by flow cytometry. Exclusively samples with >70% CD14⁺ cells were used to generate PBMDCs. Briefly, the cells (1×10^6 /ml) from each donor were cultured in culture medium described above supplemented with 200 IU/ml of granulocyte-macrophage colony-stimulating factor (rh GM-CSF, CS-C1038, Cellsystems, Germany) and 100 IU/ml of interleukin-4 (rh IL-4, CS-C1064, Cellsystems, Germany) for 5 days (37°C , 5% CO_2) in 6-well culture plates, unless otherwise indicated. The IL-4 cytokine activity was determined by the manufacturer using dose dependant stimulation in a standard proliferation assay of TF-1 cells in direct comparison with WHO standard (88/656, NIBSC). Their result (<0.2 ng/ml) indicates a specific activity of 5×10^6 IU/mg (Kitamura et al., 1989).

2.4. Chemical treatment of PBMDCs

After 5 days of differentiation culture, cells were harvested, counted and their CD86 phenotype measured by flow cytometry. Samples with <50% CD86⁺ cells were then seeded in 24 well plates (7.5×10^5 cells/ml; 0.4 ml per well) and the test chemicals added at the desired concentrations. After 48 h (37°C , 5% CO_2), unless otherwise noted, the CD86 phenotype was determined by flow cytometry. For each donor, a negative control (RPMI 1640 medium), a positive control (10 μ M DNCB) and a functional control (100 ng/ml LPS) were included in the test set and analyzed in parallel.

2.5. Flow cytometry analysis

5×10^5 cells were prepared for flow cytometric analysis by centrifugation (16,000g for 10 s) in PBS. Cells were resuspended in 100 μ l of PBS and incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibody according to the manufacturer's instruction. The following antibodies were used: anti-CD86-PE: BD555658; anti-CD14-PE: BD555807; anti-CD14-FITC: BD345784; all from BD Biosciences, Basel, Switzerland. 7-aminoactinomycin (7-AAD, BD559925; BD Biosciences, Basel, Switzerland) was used to determine dead cells (Schmid et al., 1992). To assess non-specific binding the isotypic controls Mouse IgG1-PE, BD555749 and Mouse IgG1-FITC, BD349051 (BD Biosciences, Basel,

Switzerland) were used as described in manufacturer's instructions. Cells were resuspended in 400 μ l of PBS (containing 0.1% sodium azide) and all samples were additionally stained with 7-AAD for 10 min before measurement. The flow cytometry analysis was performed on a FACS Canto II (Beckton Dickinson, San Jose, CA, USA) flow cytometer. For each sample 10,000 cells were analyzed. Gates for lymphocytes and debris exclusion were set based on forward and sideward scatter signals. Dead cells were assessed based on 7-AAD signals (Schmid et al., 1992) and excluded from the CD86 analysis. Data analysis was performed with the FACSDiva software (Beckton Dickinson, San Jose, CA). Changes of surface marker expression after treatment were calculated as difference to untreated controls (Δ CD86 = %CD86+ cells in substance treated sample – %CD86+ cells in untreated sample). Statistical analysis was performed with STATISTICA 8.0 (Statsoft, Tulsa, OK, USA).

3. Results

3.1. Moderate interleukin-4 concentrations are sufficient for inducing the differentiation of peripheral blood monocytes into functional peripheral blood monocyte-derived dendritic cells

Phenotypical modification of the isolated blood monocytes from a CD14^{low} CD14^{high} phenotype to a CD14^{high} CD14^{low} phenotype typical of immature PBMDs in response to increasing IL-4 concentrations (0–300 U/ml (see unit definition in the materials and methods section)) was considered as the critical parameter for evaluating the cell differentiation status (Fig. 1A). After 5 days of culture in presence of 200 U/ml of GM-CSF and the indicated IL-4 concentrations (Fig. 1B), the expression of CD14 and CD14 as differentiation markers and of CD86 as an activation marker were analyzed by flow cytometry. IL-4 concentrations above 3 U/ml induced the differentiation of monocytes to PBMDs. No further modification of the CD14^{high} CD14^{low} phenotype was detected when IL-4 concentrations from 10–300 U/ml were used (Fig. 1B). Surprisingly, an increase in CD86 expression was observed when IL-4 concentrations <10 U/ml were applied. Therefore we evaluated the influence of IL-4 concentrations on the modulation of CD86 expression in response to hapten exposure. PBMDs differentiated with IL-4 concentrations of 0–1000 U/ml were exposed to 10 μ M DNCB (a concentration known to induce a relevant increase in CD86 expression on responsive PBMDs) or medium and analyzed for CD86 expression after 48 h (Fig. 2A). The up-regulation of CD86 expression in response to hapten exposure decreased at IL-4 concentrations <100 U/ml but was not

significantly affected at IL-4 concentrations between 100 and 1000 U/ml (Fig. 2B). These results indicate that an IL-4 concentration $\geq$ 100 U/ml is required to obtain PBMDs susceptible to activation through hapten exposure. Therefore an IL-4 concentration of 100 U/ml was used in all further experiments.

3.2. A 48 h exposure to the hapten is required for an optimal modulation of the CD86 expression

To define the optimal exposure period, immature PBMDs were exposed to 100 μ M nickel sulfate, 10 μ M DNCB and 100 μ M SDS for 24, 48 or 72 h. At the end of the exposure period, the expression of the cell surface markers HLA-DR, CD1a, CD14, CD83 and CD86 was measured by flow cytometry. Significant and reliable differences were detected in the expression of CD86 only. Whereas 24 and 48 h exposure times induced similar and reproducible CD86 expression, the 72 h incubation time resulted in a slightly reduced but highly variable CD86 expression (Fig. 3).

3.3. Pooling of PBMDs is not necessary due to the limited donor to donor variability in the basal expression of cell surface markers

The magnitude of donor to donor variations in the basal expression of CD86 was measured by flow cytometry on differentiated PBMDs (for differentiation conditions see Section 2) isolated from 172 donors over a period of 3 years. This study revealed a median of 25.7% of CD86 positive cells with first and third quartile values of 16.3 and 37.6, respectively (Fig. 4). To further evaluate the influence of the experimental conditions (e.g. lab personnel, equipment, etc.), we monitored the basal CD86 expression of differentiated PBMDs obtained in two different laboratories, isolated by different personnel over an 9 months period (31–38 donors each). The median basal CD86 expression of the differentiated PBMDs varied in a range from 21.9% to 27.2% with first and third quartiles of 12.65 and 32.95 to 18.2 and 43.8, respectively. Interestingly the collaborating laboratory (Henkel) using an unrelated buffy coat supplier, their own laboratory staff and different equipment, has obtained very similar values for 90 donor samples (median basal CD86 positive population: 28.2% with first and third quartiles of 22% and 42.8%, respectively). All together, the basal CD86 expression of differentiated PBMDs obtained from 397 different donors was investigated and revealed a surprisingly low variability of the overall median CD86 expression (26.7% with a 1st quartile at 17.7 and a 3rd quartile at 40.5). Taken together these data indicate a limited donor to donor variation for CD86 basal expression reflect-

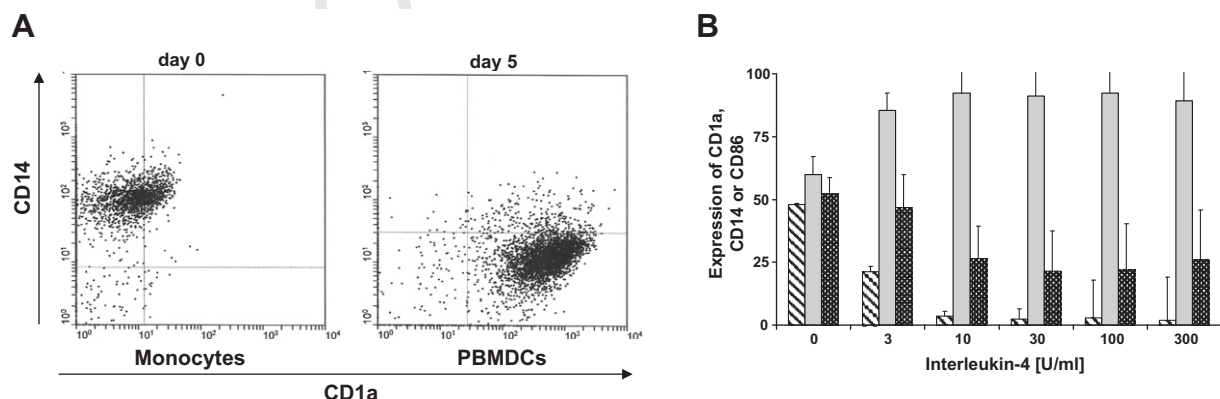


Fig. 1. Phenotypic characterization of monocytes and PBMDs by flow cytometry. (A) Dotplots of CD14 (y-axis) and CD1a (x-axis) measurements on monocytes at day 0 (left) and on PBMDs differentiated for 5 days with 100 U/ml IL-4 and 200 U/ml GM-CSF (right). (B) Expression of the surface markers CD14 (striped), CD1a (grey), and CD86 (dotted) on PBMDs differentiated for 5 days with the indicated concentration (X-axis) of IL-4. Means (bars) and SD (whiskers) of the results obtained with the PBMDs derived from eight different donors are indicated.

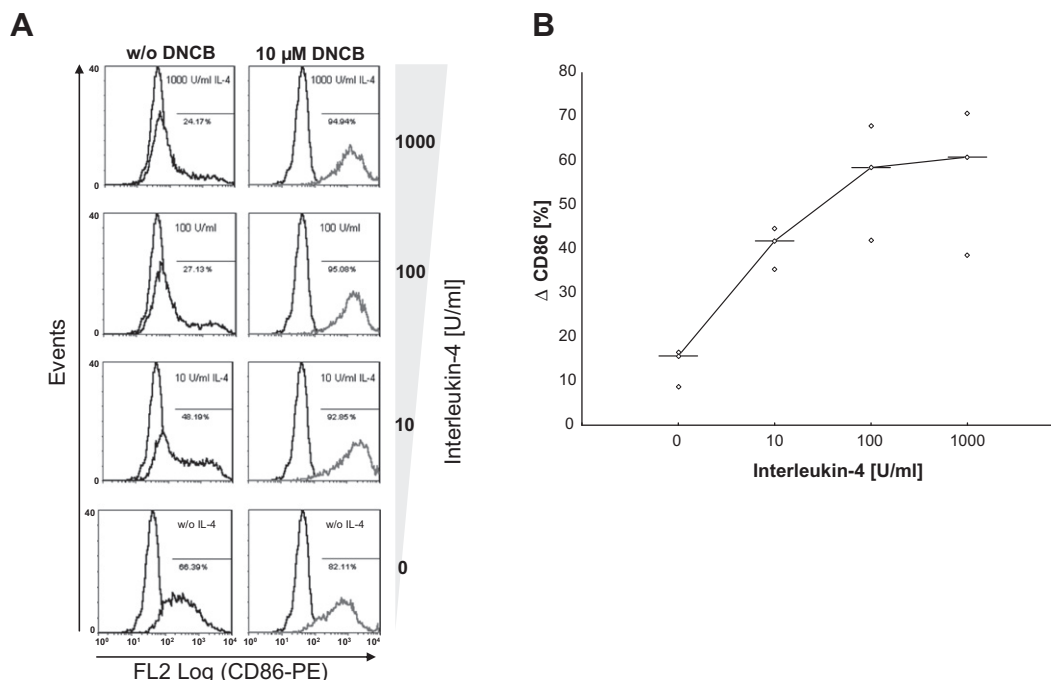


Fig. 2. Flow cytometric analysis of DNCB induced modulation of CD86 expression on PBMDs obtained with increasing IL-4 concentrations. (A) Histograms obtained for CD86 (black line) and isotype control (grey line) on PBMDs differentiated with the indicated IL-4 concentrations. Left histograms: unexposed cells. Right histograms: cells exposed to 10 μM DNCB for 48 h. (B) %ΔCD86 obtained on PBMDs differentiated with increasing IL-4 concentrations. Median (horizontal bars) of the relative increases in CD86 expression measured on PBMDs (obtained from two different donors) differentiated with the indicated IL-4 concentration (X-axis) and exposed to 10 μM DNCB. The individual values are indicated as circles.

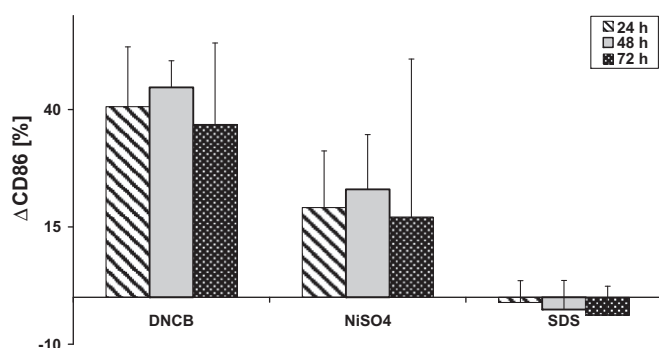


Fig. 3. Modulation of the CD86 expression as a function of the chemical exposure time. Flow cytometric measurement of the modulation of the CD86 expression (ΔCD86 [%]) on PBMDs exposed for 24, 48 and 72 h to 10 μM DNCB, 100 μM NiSO4 or 100 μM SDS. Mean values (boxes) and SD ($n = 7$) are indicated.

rial. However, differences in CD86 expression modulation upon chemical exposure cannot be excluded. These differences have been evaluated in our laboratory for a few chemicals and are very limited (unpublished results).

3.5. Relative cytotoxicity induced by test chemical exposure should be limited to 20% to avoid artifacts

To measure the influence of the presence of dead cells in the test mixture, different proportion of sonicated PBMDs were added to a constant number (2×10^6) of PBMDs and then incubated for 48 h. PBMDC activation was then evaluated by measuring the CD86 positive population as indicated in the methods section. A significant increase of CD86 positive cells was induced when the proportion of dead cells exceeded 20% (Fig. 6). The maximum acceptable relative cytotoxicity value compatible with our test protocol has thus been considered to be 20%.

3.6. Identification of sensitizers by measurement of CD86 expression

To evaluate the predictive capacity of this optimized PBMDC-based sensitization assay, a test set representing different classes of chemical was defined and evaluated. This set comprises the known contact sensitizers 2,4-dinitrochlorobenzene (DNCB), glutaraldehyde, cinnamic aldehyde, nickel sulfate, α-hexylcinnamic aldehyde, eugenol and hydroxycitronellal and the non-sensitizers, 4-hydroxybenzoic acid, dimethylphthalate, ascorbic acid, sodium dodecyl sulfate (SDS) and benzalkonium chloride.

As a first step, the cytotoxicity of the chemicals included in the test set was evaluated within a large concentration range limited only by their solubility. The concentration inducing a 20% cytotoxicity was first determined. The final test range was then defined by choosing a minimum of 4 lower and one higher test concentrations. PBMDs were then exposed for 48 h to the chosen test chem-

ing an unexpected robustness. To further limit possible sources of variations, samples containing a proportion of CD86 positive cells >50% are excluded from any further analysis.

3.4. Monocytes isolated by CD14 positive selection or negative depletion display similar CD86 basal expression

The influence of the monocyte isolation procedure (negative or positive CD14 selection) was investigated by comparing the CD86 basal expression on PBMDs derived from monocytes obtained through both selection methods. Briefly, buffy coats obtained from ten different donors were split into two aliquots each and separately processed either with a negative depletion or a CD14 positive bead selection. The comparison of the basal CD86 expression did not reveal any significant difference between the two groups (Fig. 5) indicating that both protocols deliver similar source mate-

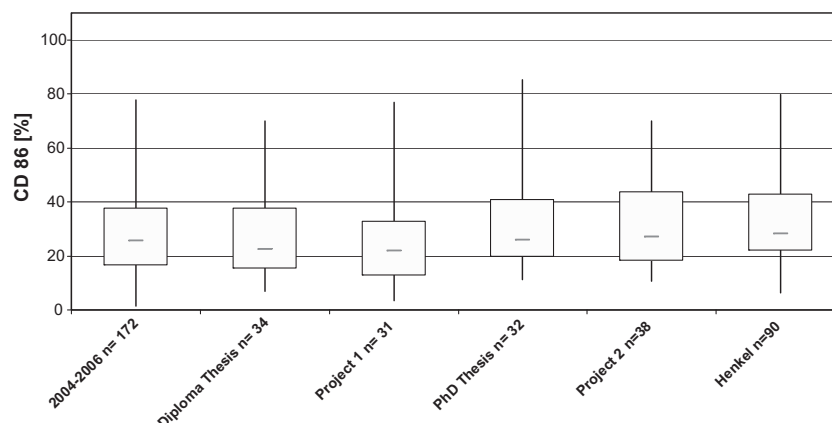


Fig. 4. Measurement of the basal CD86 expression on differentiated PBMDs obtained from 397 different donors. Flow cytometric measurement of the percentage of CD86 positive cells. Boxplot with median (horizontal ligne), 1st and 3rd quartile (box) and outliers (whiskers) indicate basal CD86 expression (% of CD86 positive cells) measured on PBMDs during various experiments including different donor numbers (as indicated on the X-axis), laboratory personnel, time periods and laboratories.

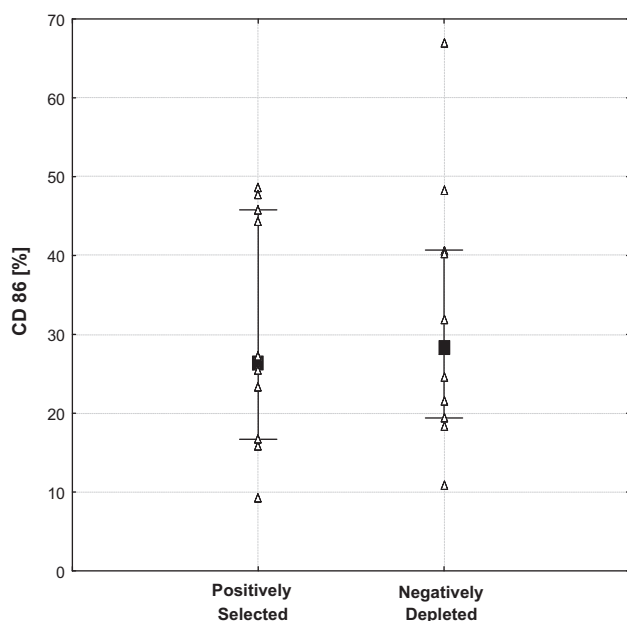


Fig. 5. Basal CD86 expression measured on PBMDs obtained by positive selection or by negative depletion. Flow cytometric measurement of the basal CD86 expression (% of the CD86 positive cells, y-axis) on PBMDs isolated by positive selection or by negative depletion from buffy coats obtained from ten different donors: single experiments (triangles), medians (black square) and 1st and 3rd quartiles (whisker) are indicated.

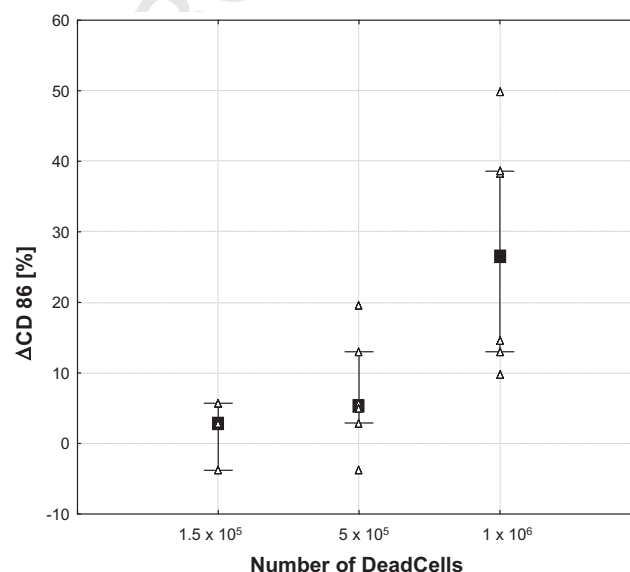


Fig. 6. Modulation of the CD86 expression in presence of increasing amount of dead cells. Induction of CD86 (ΔCD86 in %, y-axis) on PBMDs mixed with different amount of lysed PBMDs (1.5×10^5 , 5×10^5 or 1×10^6 cells) corresponding to 7%, 20% and 33% of dead cells in the assay. Single experiments (triangles), medians (black square) and 1st and 3rd quartiles (whisker) are indicated.

ical concentration range and then analyzed by flow cytometry for CD86 expression. A minimum of five independent donors was used. Each donor was tested in parallel with LPS as a functional control, DNCB as a positive control and culture medium as negative control. The percentage of CD86 positive cells is determined for each test sample and the difference between the results obtained for the treated sample and the corresponding negative control (ΔCD86) computed (Fig. 7). This ΔCD86 reflects the increase in the proportion of CD86 expressing cells induced by the test chemical. A ΔCD86 >20% obtained within the acceptable cytotoxicity domain (cytotoxicity <20%, see above) was considered as a relevant increase and classified the substance as a sensitizer. Table 1 indicates the known sensitizing category (according to LLNA), the PBMDC test results (test concentration inducing a >20% increase in ΔCD86/test concentration inducing a >20% in cytotoxicity) and the PBMDC classification obtained for each chemical in the test set.

4. Discussion

The aim of this study was to develop a stable and reproducible *in vitro* assay to be included in a test battery for the identification of chemicals with skin sensitizing potential. The described protocol is based on human PBMDs and the flow cytometric measurement of CD86 expression as an activation marker. The decision to use PBMDs as cellular system instead of cell lines was based on the fact that PBMDs are true human-like cells that are mainly free of genomic degeneration (e.g. deregulated oncogenes or signalling pathways) and display most of the normal physiological and metabolic capacity of functional DCs. Using a limited set of test chemicals, this assay was able to clearly discriminate sensitizing from non-sensitizing chemicals. This simple and stable protocol has been obtained after a comprehensive review, evaluation and optimization of the existing protocols. The first step concerned the re-

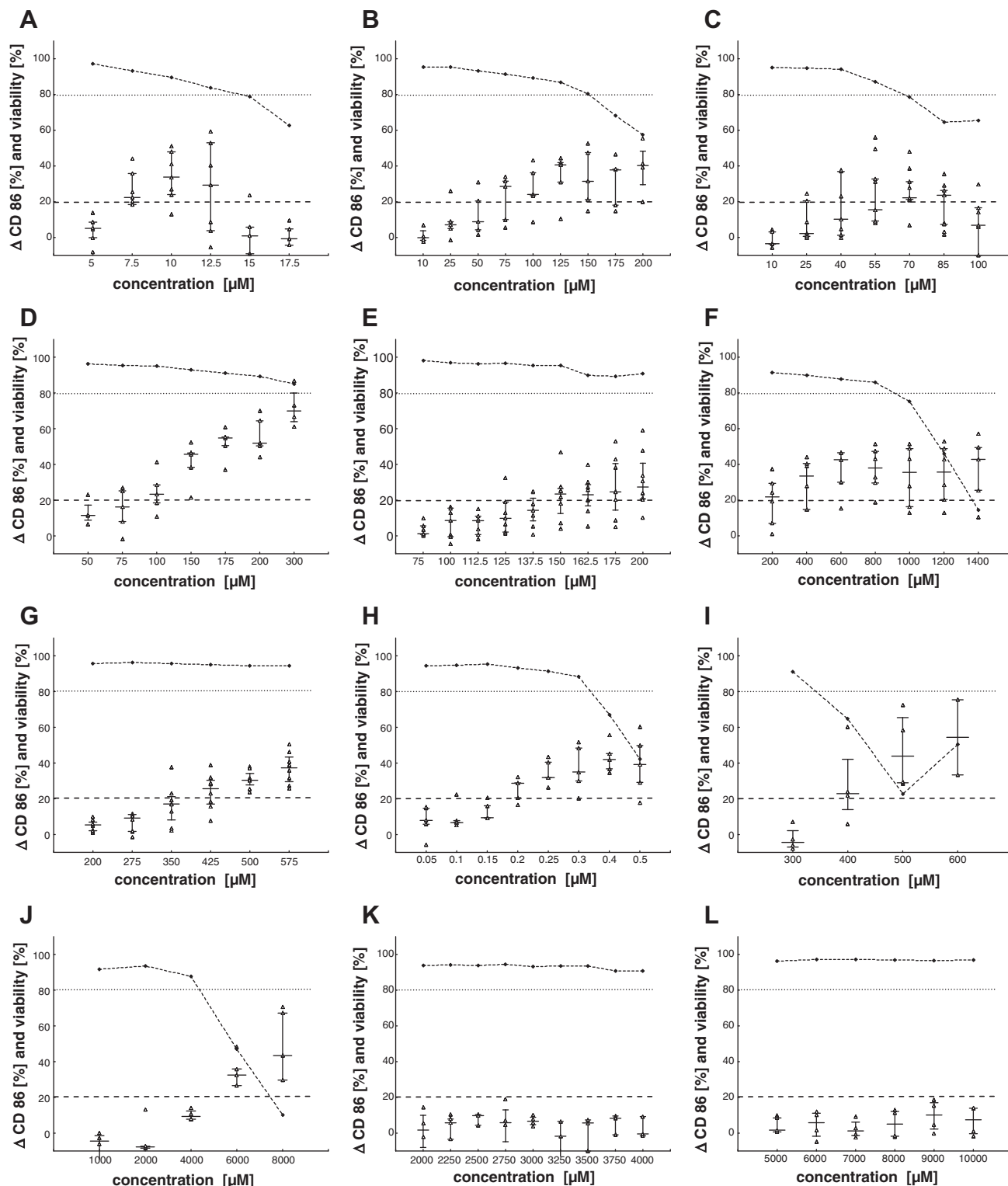


Fig. 7. Response of the PBMDc assay to sensitizers and non-sensitizers Flow cytometric measurement of the modulation of CD86 expression (%ΔCD86) on PBMDc cells obtained from a minimum of five donors exposed for 48 h to the indicated concentrations (X-axis) of test chemical. The figures A – L indicate the results obtained for seven sensitizers ((A) 2,4-dinitrochlorobenzene (DNCB), (B) glutaraldehyde, (C) cinnamic aldehyde, (D) nickel sulfate, (E) α-hexylcinnamic aldehyde, (F) eugenol and (G) hydroxycitronellal) and five non-sensitizers ((H) benzalkonium chloride, (I) sodium dodecyl sulfate (SDS), (J) ascorbic acid, (K) dimethylphthalate and (L) 4-hydroxybenzoic acid). Triangles indicate each single measurement. The horizontal bars correspond to the medians and the whiskers to the 25% and 75% quartiles. Corresponding cell viability is indicated by dotted lines. Unexposed cells were analysed in parallel in order to compute the %ΔCD86 and viability values.

evaluation of the optimal IL-4 and GM-CSF concentrations needed to obtain a differentiation of CD14^{low} CD14^{high} peripheral blood monocytes into CD14^{high} CD14^{low} immature PBMDc cells. The concentrations used, mainly that of IL-4, varied widely in the published protocols (Degwert et al., 1997; Aebj et al., 2004; Staquet et al., 2004). Moreover, the unit definition has changed between 1997 and now! Therefore we investigated the expression of two differentiation markers after incubation with various IL-4 concentrations

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Table 1

Sensitizing category, PBMDc test results and the PBMDc classification for all tested chemicals.

Test substance	Sensitizer category	20% Δ CD86 (concentration in μ M)	20% Cytotoxicity (concentration in μ M)	PBMDc classification
DNCB	Extreme	<7.5	15	Sensitizer
Glutaraldehyde	Strong	75	150	Sensitizer
Cinnamic aldehyde	Moderate	<70	70	Sensitizer
Nickel sulfate	Moderate	<100	300	Sensitizer
α -Hexylcinnamic aldehyde	Weak	<150	200	Sensitizer
Eugenol	Weak	<200	1000	Sensitizer
Hydroxycitronellal	Weak	425	575	Sensitizer
Benzalkonium chloride	NS	0.2	0.4	Sensitizer
Sodium dodecyl sulphate (SDS)	NS	400*	<400	Not sens
Ascorbic acid	NS	6000*	>4000	Not sens
Dimethylphthalate	NS	Not detected	Not detected	Not sens
Dihydroxybenzoic acid	NS	Not detected	Not detected	Not sens

NS: Not sensitizing according the LLNA classification.

* Cytotoxicity >20%.

trations (using current unit definition). The modulation of the activation marker CD86 in response to hapten exposure was measured as well. IL-4 (100 U/ml) was found to be the optimal concentration for differentiating the monocytes into functional PBMDcs suited to our protocol needs. Interestingly IL-4 concentrations below 100 U/ml resulted in an elevated CD86 basal expression that did not allow a sufficient dynamic range for the modulation of the CD86 expression after exposure to sensitizers. This may result from a macrophagic differentiation pathway.

The wide range of exposure duration to chemicals described in the existing literature (30 min to 96 h; (Degwert et al., 1997; Reuter et al., 1997; Aeby et al., 2004; Staquet et al., 2004) was a clear indication that this parameter needed further evaluation and optimization. The effect of 24, 48 and 72 h chemical exposure times on CD86 expression was thus measured.

24 and 48 h exposure durations induced relevant and reproducible increases in CD86 expression whereas 72 h led to a highly variable and slightly decreased CD86 response. Forty-eight hours was thus chosen as the best compromise and has been used in all further experiments. Our data also support the use of 24 h as an alternative and adequate exposure period.

Donor to donor variation in the basal expression of cell surface markers such as CD86 was often stated as a critical point against the use of PBMDcs for the development of *in vitro* sensitization assays (Aiba et al., 1997; Basketter and Maxwell, 2007). A major finding of the present study is that this suspected donor to donor variability is quite limited! This important finding was obtained by measuring the basal CD86 expression on PBMDcs obtained from more than 397 different donors, purified in different times and places (two different laboratories) by technicians using different equipment. A surprising low variability of the overall median CD86 expression (26.7% with a 1st quartile at 17.7 and a 3rd quartile at 40.5) was observed. It is the first time that this biological parameter is investigated so extensively and our results are in marked contrast with previous studies performed on a limited number of donors indicating a high donor to donor variability (Aiba et al., 1997; Basketter and Maxwell, 2007). However, to further reduce possible source of variability, a strict criteria for selecting the cells (exclusion of PBMDcs with elevated (>50%) CD86 expression) has been added. The limited variability of the cells and the inclusion of a selection criterion allowed us to perform experiments with cells obtained from a single donor instead of pooling cells isolated from multiple donors as proposed in other protocols (Degwert et al., 1997; Aeby et al., 2004).

A further major difference between the published protocols concerns the CD14 positive monocyte isolation process. Either positive (selection of the monocytes by antibody binding to the CD14 receptor) or negative (depletion of unwanted cells) isolation proce-

dures have been described (Pickl et al., 1996; Hu et al., 2004). To avoid possible cell activation through the interaction with the CD14 receptor, a negative selection (depletion) approach was chosen. However, a comparison of the two methods applied on the same blood samples did not show any relevant difference in CD86 expression. We concluded that both procedures have a very similar effect on the activation state of the PBMDcs and yield similar source material. However, in order to keep open the possibility of an assay extension with a mixed lymphocyte reaction, we decided to use only the CD14 positive selection procedure.

A certain level of cytotoxicity is required by most cell based sensitization protocols for inducing measurable cell activation. Most *in vitro* sensitization test protocols published so far indicate the maximum acceptable relative cytotoxicity value that should be induced by exposure to the test chemical. These values range from 15% to 50% (Degwert et al., 1997; Aeby et al., 2004; Ade et al., 2006; Ashikaga et al., 2006; Sakaguchi et al., 2006). The highest acceptable concentration for the analyzed test chemical is then usually defined as the one inducing that maximum relative cytotoxicity value. But toxic effects may also induce artefacts (false positives). The maximum acceptable level of cytotoxicity thus represents a crucial parameter that should be carefully analyzed and defined. DCs can be activated by danger signals which are partly due to the release of certain cell components by dying or dead cells (Gallucci and Matzinger, 2001; Nestle et al., 2009). DCs may also react to the cytokines secreted by cells under physiological stress. It was thus important to measure precisely the amount of dead cells compatible with our assay system. The chosen approach was to measure the influence of the presence of dead cells debris in the test setup. Significant cell activation was indeed measured in presence of cell lysate corresponding to 33% of dead cells. However, an acceptable PBMDcs activation level (median of 5.35%) was measured in the presence of the equivalent of 20% of dead cells. We could thus set the upper limit for the amount of dead cells compatible with our assay protocol to 20%.

To characterize the predictive capacity of this optimized PBMDc-based sensitization assay, a test set representing different classes of chemicals was defined and evaluated. Chemicals inducing a Δ CD86 >20% (See Section 3 for definition) within the acceptable cytotoxicity range (<20%) were considered as sensitizers. The results obtained with this assay configuration were then compared to the corresponding LLNA classification (Basketter et al., 1994, 2001; Kimber et al., 1995, 1998; Loveless et al., 1996; Hilton et al., 1998; Dearman et al., 2001). A correct classification (sensitizer/non-sensitizer) was obtained for all chemicals but benzalkonium chloride. Furthermore, potency information could be obtained by interpolating the test concentration inducing a Δ CD86 of 20% (EC20). The following EC20 values were calculated

(See Table 1 for a comparison): DNCB: 7.2 μM ; glutaraldehyde: 64.1 μM ; cinnamic aldehyde: 65.1 μM nickel sulfate: 88.3 μM . These relatively low EC20 values would classify the corresponding chemicals as strong allergens. The much higher EC20 values obtained for α -hexyl cinnamic aldehyde (154.3 μM) and the weak allergens eugenol (183.5 μM) and hydroxycitronellal (375 μM) allowed to classify these chemicals as weak allergens. This correlates with their corresponding LLNA classification (Basketter et al., 1994; Loveless et al., 1996). Benzalkonium chloride that is discussed to be a false positive in other assays (e.g. LLNA, see Gerberick et al. (1999)) also appears to be highly positive in this assay (EC20: 0.2 μM). On the other hand, SDS, also considered as a probable false positive in several *in vitro* assays was clearly negative in this one. Interestingly, SDS and ascorbic acid did induce a $\Delta\text{CD86} > 20\%$ but at cytotoxic concentrations only. This may result from the proposed activation effects (e.g. dead or dying cells releasing a cocktail of danger signals in the culture medium, see Section 3) again stressing the importance of a clearly defined cytotoxicity limit. Dimethylphthalate and 4-hydroxybenzoic acid, both negatives in the LLNA, were clear negatives in this PBMD assay. Even at concentrations $> 4 \text{ mM}$ neither CD86 induction nor cytotoxicity could be measured.

In this paper we describe the successful development of a stable and robust test protocol based on human PBMDs for the discrimination of sensitizing from non-sensitizing substances. The use of human PBMDs as an alternative to available cell lines was dictated by the inherent issues (phenotypical and genotypical instabilities, deficiencies in metabolism, variable performance depending on passage (Sakaguchi et al., 2010)) linked with the use of cell lines. The critical test parameters (monocyte isolation procedure, cytokine/chemokine concentrations, treatment conditions, cytotoxicity threshold) were first identified and optimized. The resulting assay was then evaluated with a test set comprising sensitizing and non-sensitizing chemicals. Its performance was established and can be considered as very promising. It offers an alternative to cell line based protocols when test conditions closer to the human *in vivo* situation are desired. Once its reliability is confirmed by a comprehensive inter laboratory ring study including the testing of a variety of additional chemicals, it may be validated and included in a test battery for characterizing the sensitizing potential of chemicals without the need of animal data.

Disclosure statement

The authors have no conflicting financial interest

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