

Original article

A loose-fit coculture of activated keratinocytes and dendritic cell-related cells for prediction of sensitizing potential

Protection against contact allergy begins with the collection of reliable data about the sensitizing potential of chemicals. Today, the local lymph node assay (LLNA) in mice is widely used to identify sensitizing substances. For several reasons, an *in vitro* assay could be preferable to animal experiments. We propose an *in vitro* test for the detection of a sensitizing potential of a chemical composed of a single layer of human nondifferentiating keratinocytes and of allogenic floating monocytes which are cocultured in serum-free medium in the presence of a cytokine cocktail. Within days, the coculture develops to an allergen-sensitive system consisting of activated keratinocytes and of mobile dendritic cell-related cells (DC-related cell). The sensitizing potential can be determined by analyzing the expression of the dendritic cell maturation marker CD86. For the model contact allergens tested so far [trinitrobenzenesulfonic acid (TNBS), phenylendiamine, and 4-aminoacetanilide], the strength of the reaction was in concordance with results from the LLNA. Sensitivity of the assay allowed testing at concentrations without general cytotoxicity. Thus, a differentiation between allergens and irritants was possible. Regarding cytokine secretion, the assay distinguished between the allergen TNBS and the Toll-like receptor ligand lipopolysaccharide. The coculture can be set up from cryopreserved cells. The assay is easy to perform and reproducible. Donor-variance is negligible. This *in vitro* assay based on a loose-fit coculture is a reasonable approach to screen for the sensitizing potential of xenobiotics and might partially replace the LLNA and other animal tests.

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The term 'sensitizing potential' describes the potential of a substance to induce allergic contact dermatitis (ACD) [see Nomenclature in Ref. 1]. The prevalent and Organization of Economic Cooperation and Development-approved assay to determine the sensitizing potential of a substance is the mouse local lymph node assay (LLNA) (2–4). The LLNA reduced numbers and burden of testing the animals as compared with its progenitor tests. However, ethically desirable would be the use of an *in vitro* assay. Further need of improvement concerns the reliability of LLNA test scores (5). The sensitizing phase

of ACD development is defined by contact allergen-induced maturation of dendritic cells to antigen-presenting cells (APC) (review in Ref. 6). Langerhans cells represent a special type of immature dendritic cells located in the epidermis (7, 8). This would suggest to use cultured Langerhans cells in an *in vitro* assay for the detection of contact allergens (review in Ref. 9), however, this is still impossible as human Langerhans cells isolated from epidermis rapidly lose their viability (10).

Human cell lines which may adequately mimic authentic dendritic cells are not available. Experiments using THP-1, KG-1, or MUTZ-3 cell lines revealed that these cells react only to extremely strong allergens at near-cytotoxic concentrations (11, 12). In addition, tumor cell lines in particular are known to tend to genetic and phenotypic instability. The price for easy handling consists in uncertainty and impaired reproducibility of test results. Recent findings using the human myeloid leukemia cell line U937 should be viewed against this background (13). To obtain alternative test cells, procedures were developed to use human precursor cells for *in vitro* generation of dendritic cells. Such precursor cells

Abbreviations: LLNA, local lymph node assay; TNBS, trinitrobenzenesulfonic acid; LPS, lipopolysaccharide; ACD, allergic contact dermatitis; TGF, tumor growth factor; KC, keratinocyte; APC, antigen presenting cell; PPD, para-phenylendiamine; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; KGM, keratinocyte growth medium; FACS, fluorescence activated cell sorting; MMP, matrix metalloproteinase; 7-AAD, 7-amino-actinomycin; moLC, monocyte derived Langerhans-like cells; DC-related cells, dendritic cell-related cells; MFI, mean fluorescence intensity; TNF, tumor necrosis factor.

may be monocytes isolated from adult blood or from leukocyte concentrates. Cytokine cocktails induce the differentiation of precursor cells to immature dendritic cell-like cells (14–17).

In vitro generated dendritic cell-like cells were intensely studied concerning their capability to detect sensitizing substances. However, generated dendritic-like cells do not suit well as sentries when cultured solitarily. Detectable concentrations of contact allergens were close to toxic ranges, and a clear cut differentiation between allergens and irritants was hardly possible (9, 18, 19). A considerable cell donor dependent variance of test scores demanded the use of cells pooled together from several donors (20, 21). Another problem in using generated dendritic-like cells is the tendency of these cells to spontaneous stimulation-independent maturation during cell culture. Inclusion of tumor growth factor- β (TGF- β ; R&D, Wiesbaden-Nordenstadt, Germany) during dendritic-cell generation results in Langerhans-like cells which do not tend to spontaneous maturation. However, in solitary culture, they also failed to deliver convincing test results (10, 22–24).

Low-molecular weight allergens are haptens, or even prohaptens, which need association with proteins to become immunogenic antigens. Keratinocytes (KC) are involved in this proteinization process. In addition, KCs may participate in allergen-induced maturation of dendritic cells by providing danger signals (25). In a cell based assay system for sensitizing potential, dendritic cells should therefore be used in combination with KCs. Human KCs can be raised to three-dimensional cell cultures including all distinctive differentiation stages from the basal to the horny layer. Such complex cell cultures are entitled skin equivalent, epidermis equivalent, organotypical skin model, or reconstructed epidermis (26, 27) and are commercially provided for use in toxicity testing [CellSystems (St. Katharinen, Germany), MatTek Inc. (Ashland, MA, USA), Phenion (Düsseldorf, Germany), SkinEthic (Nizza, France), to name a few).

An *in vitro* system for sensitizing potential based on a reconstructed epidermis should include dendritic cells. However, if excised epidermis is subjected to organic culture, the Langerhans cells will emigrate spontaneously into the culture medium (28). It is not known whether Langerhans cells are continuously connected to suprabasal KCs, or whether immature Langerhans cells are already mobile. Only a few studies reported on multilayered skin equivalents into which dendritic cell precursors could be integrated coherently and which could then be differentiated (29, 30; patent EP 0789074). There is no approved test system for sensitization available based on a multilayered skin equivalent with integrated dendritic cells. Problems arose from considerable cell donor dependent variance of test scores, a lack of coherence between the cells in the test kit, and from unwanted influences of the fibroblasts. We propose an *in vitro* test kit for sensitization, which is composed of a single layer

of human nondifferentiating KCs and of allogenic floating monocytes, which are cocultured in serum-free medium in presence of interleukin-4 (IL-4), GM-CSF, and TGF- β . Thus, the loose-fit coculture develops into an allergen-sensitive system consisting of activated KCs and of mobile DC-related cells. We tested the performance of this new assay (called loose-fit coculture-based sensitization assay [LCSA]) by using 2,4,6-trinitrobenzenesulfonic acid (TNBS) and para-phenylenediamine (PPD; Sigma, Deisenhofen, Germany) which are classified in LLNA as strong sensitizers (13, 31) and the textile dye cleavage products 4-aminoacetanilide, a weak sensitizer in LLNA (32), and 2-amino-*p*-cresol, a stronger sensitizer as compared with aminoacetanilide (32).

Materials and methods

Media and reagents

Cells were usually cultured in serum-free KC growth medium 2 [keratinocyte growth medium-2 (KGM-2); PromoCell, Heidelberg, Germany] supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Biochrom, Berlin, Germany). For some experiments, RPMI 1640 with ultraglutamine 1 (Cambrex, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% heat inactivated fetal calf serum (FCS) (Biochrom) was used. 2,4,6-TNBS (Sigma) was dissolved in sterile pyrogen-free water (DeltaSelect, Dreieich, Germany). Paraphenylenediamine was dissolved in ethanol abs (Merck, Darmstadt, Germany). Final concentrations of ethanol in cell culture medium did not exceed 1%. 4-Aminoacetanilide (Aldrich, Deisenhofen, Germany) and 2-Amino-*p*-cresol (2-Amino-4-methylphenol; Aldrich) were dissolved in dimethyl-sulfoxide (DMSO Hybrimax, Sigma). Final concentrations did not exceed 1%. Sodium dodecyl sulphate (SDS Ultra) was obtained from Fluka (Deisenhofen, Germany), and lipopolysaccharide (LPS) (*Escherichia coli* serotype 0128:B12) was from Sigma.

Cryopreservation of primary human keratinocytes

Human skin from healthy donors was received in adherence to the Declaration of Helsinki principles as residual material from plastic surgery with local ethics committee approval. After incubation in PBS containing 2 U/ml of dispase I (Roche, Mannheim, Germany) for 18 h at 4°C, epidermal sheets were stripped off the dermal layer and dissociated in PBS containing 0.25% trypsin (Biochrom) and 0.01% DNase (Roche, Mannheim, Germany) for 15 min at 37°C. Single cell suspensions were obtained by passing through a 40- μ m cell strainer (BD, Heidelberg, Germany). Cells were washed in PBS, resuspended in KGM-2 and seeded on Costar Cell Culture Flasks (Corning, Schiphol-Rijk, The Netherlands) at a density of $2\text{--}5 \times 10^5$ cells/cm². Cells were cultured until confluence with medium changes on alternate days. Finally, KCs were harvested by trypsinization and cryopreserved in FCS with 10% DMSO (Hybrimax, Sigma) at a density of 6×10^6 cells/ml.

Cryopreservation of human monocytes and PBMC

Peripheral blood mononuclear cells (PBMC) were enriched with fresh buffy coat preparations (DRK Blutspendedienst, Berlin, Germany) by density centrifugation on Ficoll-Paque (Biochrom). Monocytes were purified from PBMC by positive selection of

CD14⁺-cells using magnetically labelled anti-CD14 antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany). Monocytes were frozen at a concentration of $2-3 \times 10^7$ cells/ml as described above for KCs. Alternatively, PBMC were frozen prior to monocyte purification.

Sensitization assay

Cryopreserved KCs were typically seeded in 12-well plates (Costar Cell Culture Cluster, Corning) at a density of $2-6 \times 10^4$ cells/cm² in KGM-2. When half-confluence was reached, cryopreserved PBMC were resuspended in KGM-2 and seeded onto KCs at a density of $1-3 \times 10^6$ cells/cm². After 2 h, nonadherent cells were washed away using warm KGM-2. Alternatively, cryopreserved CD14-isolated monocytes were seeded onto KCs at a density of $2-5 \times 10^5$ cells/cm². Then, IL-4, GM-CSF (both from Immunotools, Friesoythe, Germany) and TGF- β 1 were added at final concentrations of 100, 100, and 10 ng/ml, respectively (day 0). On days 2 and 4, GM-CSF and TGF- β 1 were added again. On day 6, probes were added. After 48 h, nonadherent cells were pipetted off the coculture, counted (Coulter Z1, Beckman-Coulter) and prepared for fluorescence activated cell sorting (FACS) analysis. Volumes of medium supernatants were determined prior to freezing at -80°C for later use in enzyme-linked immunosorbent assay (ELISA).

FACS analysis

Antibody staining was measured in a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Upregulation of CD86 expression was calculated by the following equation: mean fluorescence intensity (MFI) CD86 in treated cells/MFI CD86 in vehicle control. 7-amino-actinomycin (7-AAD; ViaProbe, BD Biosciences) was used to exclude/determine dead cells. The following conjugated antibodies were used with their corresponding isotypes: FITC anti-CD11c (mouse IgG1, clone BU15, Serotec, Düsseldorf, Germany), FITC anti-CD1c (mouse IgG2a, clone AD5-8E7, Miltenyi Biotec), FITC anti-CD14 (mouse IgG2a, clone M5E2, BD Biosciences), FITC anti-human leukocyte antigen-DR (HLA-DR) (mouse IgG2a, clone G46-6, BD), PE anti-Langerin (CD207; mouse IgG1, clone DCGM4, Beckman-Coulter, Krefeld, Germany), PE anti-CD86 (mouse IgG1, clone FUN-1, BD Biosciences), APC anti-CD1a (mouse IgG1, clone HI149, BD Biosciences).

ELISA

Matrix metalloproteinase-9 (MMP-9) and cytokines were detected in cell culture supernatants using DuoSet ELISA kits from R&D Systems (Wiesbaden-Nordenstadt, Germany). Results were calculated for 10^6 harvested cells and corrected in regard to exact volumes of cell culture media.

Results

Characterization of loose-fit KC/monocyte coculture

Peripheral blood mononuclear cell were seeded onto half-confluent KC cultures. Monocytes were enriched by selective adherence to KC or to the plastic surface. During coculture in presence of cytokines, monocytes detached, and a considerable part of them changed their morphology into dendritic cell-resembling cells. However, dendrites were less pronounced as compared with

monocyte derived Langerhans-like cells (moLC) generated in RPMI/10% FCS [not shown; (22)]. Nonadherent cells were harvested and analyzed by FACS (Fig. 1A). Scatter analysis shows the cellular composition of harvested cells. The cells could be divided into two main populations (R1, R2) plus some remaining lymphocytes. Practically no KC were harvested from the coculture as analyzed cells were almost entirely CD11c⁺ myeloid cells (not shown). All cells had lost the typical monocyte antigen CD14 (not shown). R1-cells contained a high proportion of 7-AAD⁺ dead cells and did not express any of the antigens CD1c, CD1a, nor Langerin (Fig. 1A). R2-cells were mostly vital and were CD1c^{dim}, CD1a⁻, and Langerin⁻ (Fig. 1A). R2-cells harvested from cocultures are further referred to as DC-related cells.

For comparison, PBMC were seeded onto the plastic surface of cell culture dishes without KC. Again, monocytes were enriched by temporary adherence. The monocytes were solitarily cultured in serum-free KGM (Fig. 1B), or RPMI/10% FCS was used (Fig. 1C). As above, cells were cultured in the presence of IL-4, GM-CSF, and TGF- β for 6 days. R2-cells from solitarily cultured monocytes in KGM were comparable with DC-related cells in regard to CD1c, CD1a, and Langerin expression (Fig. 1B). In difference, R2-cells from solitarily cultured monocytes in RPMI/10% FCS (moLC) were CD1c⁺, CD1a⁺, and Langerin⁺ (Fig. 1C). Activation of KC, monocytes, and of cocultures were analyzed by determination of secretion of the MMP-9 (33). Solitarily cultured KC but not solitarily cultured monocytes secreted MMP-9 after cytokine cocktail treatment (Fig. 1D). In cocultures, MMP-9 secretion was further increased (Fig. 1D). The amount of MMP-9 secretion was not influenced by addition of the allergen TNBS to the cell cultures (Fig. 1D).

The allergenic effects of TNBS were detected by the coculture only, and detection was cell-donor insensitive

CD14-sorted monocytes were thawed (Fig. 2A,B) and were either cultured together with KC (Fig. 2A), or alone (Fig. 2B). In an alternative setup of the test kit, temporary adherence for enrichment of monocytes was used. Cryopreserved PBMC were seeded either onto half-confluent KC (Fig. 2C), or onto the plastic surface of cell culture plates (Fig. 2D). For all groups, serum-free KGM was used as cell culture medium. At day 6 of coculture, TNBS at a final concentration of 100 μM was added, or no addition was applied. After further 48 h, nonadherent cells were harvested and analyzed by FACS (Fig. 2). Scatter analyses show the cellular composition of harvested cells. Dendritic cell-related cells are marked by rectangular gates and were further analyzed concerning expression of the myeloid cell marker CD11c and of the dendritic cell maturation marker CD86. The MFI of CD86 was increased only in TNBS treated KC/monocyte cocultures but not in solitarily cultured monocytes (Fig. 2A-D). The monocyte enrichment techniques

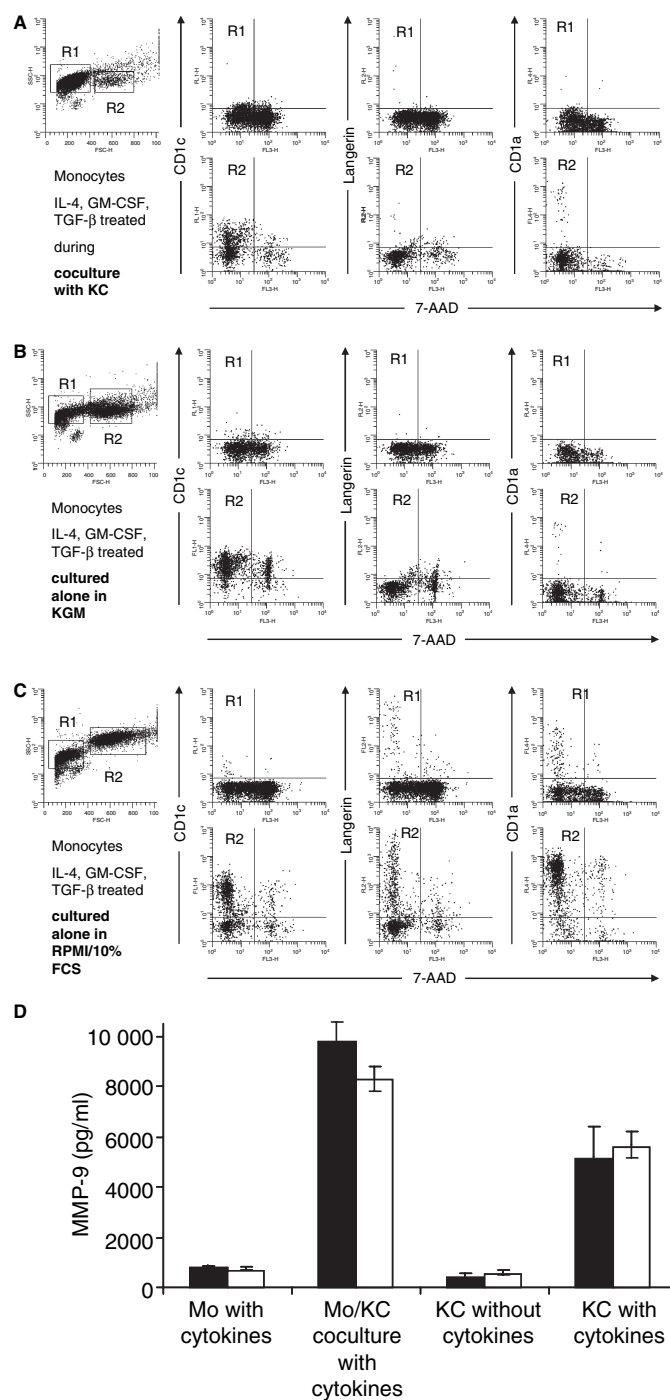


Figure 1. Serum-free cytokine-treated keratinocyte (KC)/monocyte cocultures develop to a system of dendritic cell (DC)-related cells and activated KC. Fluorescence activated cell sorting (FACS) analysis of cocultures and of solitarily cultured monocytes (A–C). Antibody-staining of cells in gates R1 and R2 is shown. Peripheral blood mononuclear cell (PBMC) were seeded on half-confluent KC cultures, and monocytes were enriched via adherence. Serum-free KGM medium was supplemented with cytokines (A). Monocytes were enriched via adherence to plastic and were cultured solitarily in cytokine supplemented keratinocyte growth medium (KGM) (B) or in cytokine supplemented RPMI/10% fetal calf serum (FCS) (C). Shown are typical results of three independent experiments. Matrix metalloproteinase-9 (MMP-9) protein was determined in media of solitarily cultured monocytes, of KC/monocyte cocultures, and of solitarily cultured KC. If present, added cytokines were interleukin-4 (IL-4), GM-CSF, and tumor growth factor- β (TGF- β). At day 6 of coculture, the allergen trinitrobenzenesulfonic acid (TNBS) was added at a final concentration of 100 μ M (white columns), or no treatment was applied (black columns). After 48 h, cell culture media were gathered for use in enzyme-linked immunosorbent assay (ELISA) (D). Shown are means ($\pm$ SD) of three independent experiments.

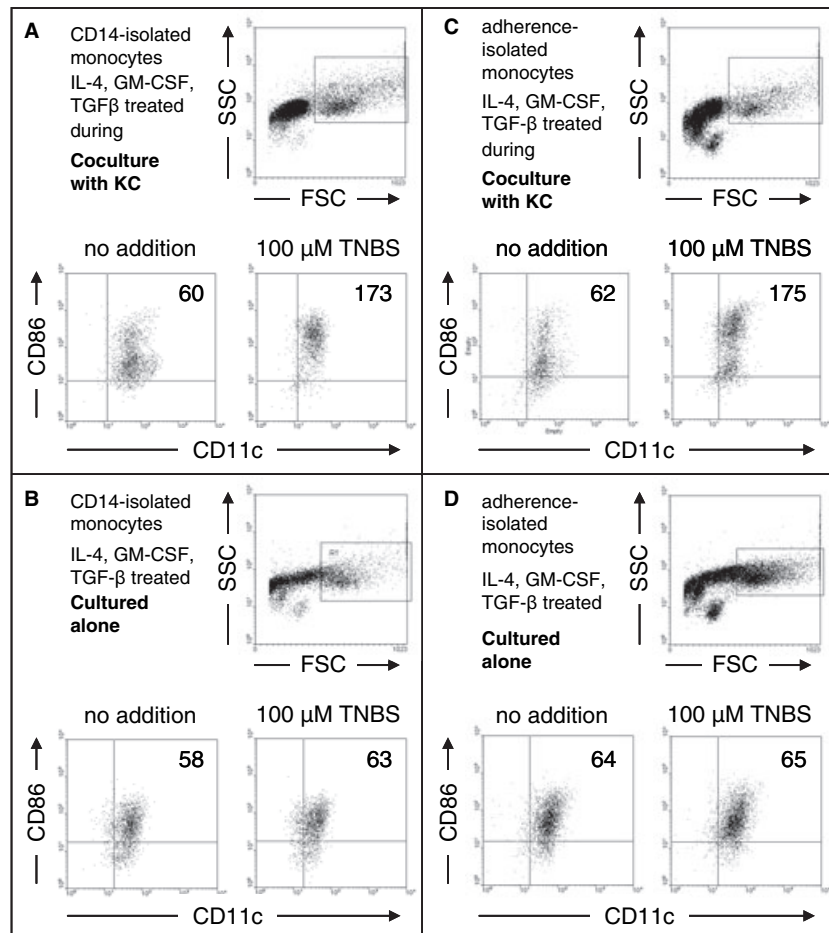


Figure 2. Monocytes treated with cytokines during coculture with keratinocytes (KC) increase CD86-expression in response to trinitrobenzenesulfonic acid (TNBS). Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by CD14-sorting (A/B) and cultured together with KC (A) or alone (B) in the presence of cytokines for 6 days. Alternatively, monocytes were enriched from PBMC by temporary adherence to KC (C) or to the plastic surface of cell culture dishes (D). Then, TNBS was added and, after 48 h, nonadherent cells were harvested and analyzed by fluorescence activated cell sorting (FACS). Cells in CD86/CD11c dot blots were gated to the regions shown in scatter analysis. Lower left quadrants correspond to isotype controls. Numbers indicate mean fluorescence intensities (MFI) of CD86 in upper right quadrants. Shown are typical results of more than 20 experiments with the use of six different cell donors.

differed mainly in the amount of residual lymphocytes and did not influence the rates of CD86-upregulation (Fig. 2A,C). For both monocyte enrichment techniques, coculturing was started when the KC had been grown to half-confluence. Experiments using fully-confluent KC cultures at the day of monocyte seeding yielded the same results (not shown). Differentiating KC cultures were not studied as the composition of the KGM medium did not allow KC differentiation.

In the next series of experiments, TNBS was added to KC/monocyte cocultures at final concentrations ranging from 12.5 to 500 μ M (Fig. 3). For both monocyte enrichment techniques, upregulation of CD86, as calculated by mean MFI-values of treatment groups divided over nontreatment controls, increased linearly up to a TNBS concentration of 100 μ M. The half-maximal upregulation of CD86 corresponded to a TNBS concentration of 40 μ M

(Fig. 3). A further increase in TNBS concentrations entered the toxic range (not shown). Worthy of mention is the stability of the results. Given are mean values ($\pm$ SD) of three independent experiments in duplicates for both monocyte isolation techniques with the use of three different blood and three different skin donors.

PPD-induced sensitization interfered with cytotoxicity

The next allergen studied was PPD, which was applied to the cocultures without using antioxidants. Para-phenylenediamine tended to induce cytotoxicity. Therefore, only 7-AAD⁺ vital cells were included into determination of CD86 expression (Fig. 4). Shown are a typical scatter-analysis and dot blots of CD86/7-AAD (Fig. 4). Already application of 31.25 μ M PPD resulted in upregulation of CD86 (Fig. 4). Para-phenylenediamine concentrations

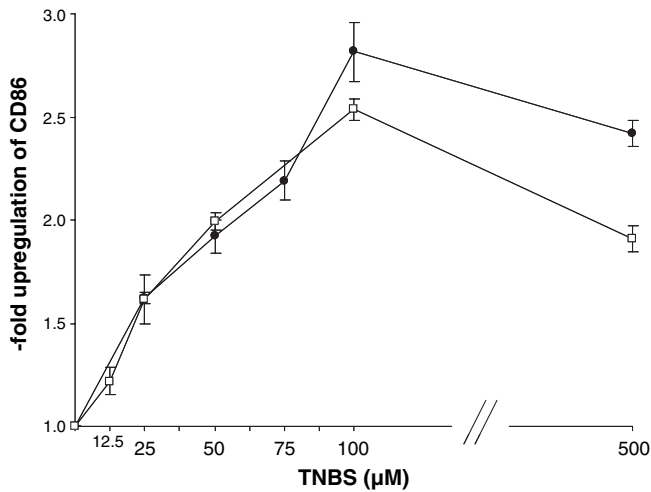


Figure 3. Rates of trinitrobenzenesulfonic acid (TNBS)-induced CD86-upregulation show only little donor-to-donor variation. CD14-isolated (□) or adherence-isolated monocytes (●) were cocultured with keratinocyte (KC) in presence of cytokines for 6 days. Then, TNBS was added at concentrations as indicated. After another 48 h, CD86-levels were analyzed as described in Fig. 2. Mean fluorescence intensities (MFI)-values were divided over nontreatment controls. Given are mean values (±SD) of six independent experiments for both monocyte isolation techniques with the use of three different blood and three different skin donors.

above 125 μM did not yield enough vital cells to allow reasonable calculation of CD86 expression.

The coculture could distinguish between disperse yellow 3 azo cleavage products

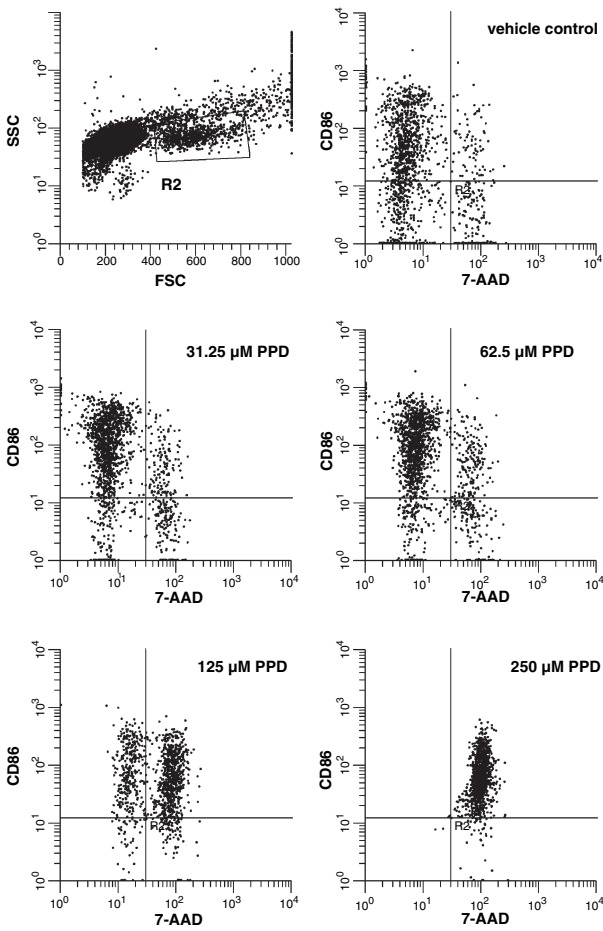
As previously determined by LLNA (32), azo cleavage products of disperse yellow 3 revealed different sensitizing potentials in the coculture test system. Upregulation of CD86 was elicited by aminocresol at lower concentrations as compared with aminoacetanilide (Fig. 5). 4-aminoacetanilide was not cytotoxic up to a tested concentration of 1000 μM. Cytotoxicity interfered with the sensitizing effects of aminocresol. 2-Amino-*p*-cresol already at 100 μM decreased the amount of vital cells to about 20% (Fig. 5).

The irritant SDS did not reveal a false sensitizing potential

The irritant SDS revealed no sensitizing potential in the coculture system at a final concentration of 20 μM (Fig. 6). At this concentration, cytotoxicity was not appreciable (not shown).

KC rendered TNBS to a danger signal detectable by serum-free cocultured DC-related cells

The upregulation of CD86 after addition of final concentrations of 100 μM TNBS and of 1 μg/ml LPS



	MFI CD86 of viable cells in R2	% of viable cells in R2
Vehicle control	53	77
31.25 μM PPD	87	79
62.5 μM PPD	81	77
125 μM PPD	64	31
250 μM PPD	-	0

Figure 4. Dose dependence of sensitizing and cytotoxic potential of para-phenylenediamine (PPD). CD14-isolated monocytes were cocultured with keratinocytes (KC) cocultures in presence of cytokines for 6 days. Then, PPD was added at concentrations as indicated without using antioxidants. After 48 h, nonadherent cells were harvested. For fluorescence activated cell sorting (FACS)-analysis of CD86/7-AAD staining, cells were gated to the region R2 as shown in scatter-analysis. Including 7-AAD-negative cells only, mean fluorescence intensities (MFI)-values of CD86 were calculated. Given are values of a typical experiment representative of four independent experiments (two different blood and two different skin donors were used).

was compared with KC/monocyte cocultures after treatment with the cytokines IL-4, GM-CSF, and TGF-β (as in experiments described above), or after treatment

	MFI CD86 of viable cells in R2	% of viable cells in R2
Vehicle control	42	84
2-Amino-p-cresol		
25 μ M	84	82
100 μ M	65	17
500 μ M	-	5
1000 μ M	-	0
4-Aminoacetanilide		
25 μ M	43	85
100 μ M	42	75
500 μ M	49	82
1000 μ M	71	76

Figure 5. Dose dependence of sensitizing and cytotoxic potential of disperse yellow 3 azo cleavage products. Monocyte/keratinocyte (KC) cocultures were set up as described in Fig. 4. At day 6, they were treated with 4-aminoacetanilide or with 2-amino-*p*-cresol at indicated doses. After 48 h, nonadherent cells were harvested. For fluorescence activated cell sorting (FACS)-analysis of CD86/7-AAD staining, cells were gated as described in Fig. 4. Including 7-AAD-negative cells only, mean fluorescence intensities (MFI)-values of CD86 were calculated. Given are values of a typical experiment representative of four independent experiments (two different blood and two different skin donors were used).

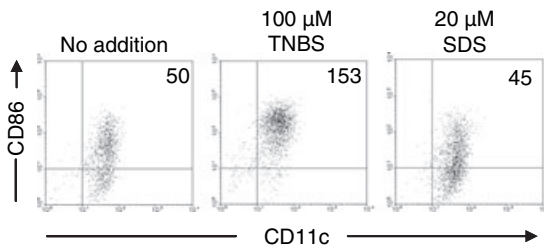


Figure 6. The coculture test system is insensitive to the irritant sodium dodecylsulphate (SDS). Monocyte/keratinocyte (KC) cocultures were set up as described in Fig. 4. Final concentrations of 100 μ M trinitrobenzenesulfonic acid (TNBS) or 20 μ M SDS were added and, after 48 h, fluorescence activated cell sorting (FACS) analysis of CD86/CD11c was performed. Cells were gated as described in Fig. 4, but no exclusion of dead cells was required. Numbers indicate CD86- mean fluorescence intensities (MFI). Shown are typical results of four independent experiments.

with IL-4 and GM-CSF only. In addition, solitary monocyte cultures were included into the comparative studies. These differed additionally in regard to the cell

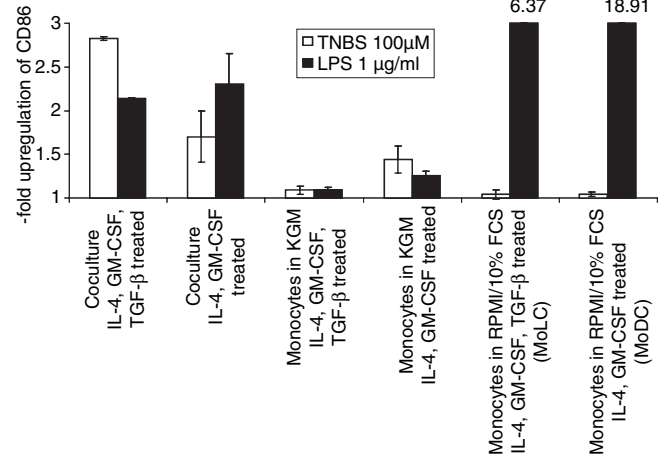


Figure 7. Keratinocyte (KC) rendered trinitrobenzenesulfonic acid (TNBS) to a danger signal detectable by serum-free cocultured dendritic cell (DC)-related cells. Cocultures were set up from KC and CD14-sorted monocytes. Serum-free keratinocyte growth medium (KGM) media were supplemented with interleukin-4 (IL-4), GM-CSF, and with or without tumor growth factor- β (TGF- β). For solitary culture of monocytes, KGM was used as medium and was supplemented with cytokines as described above, i.e. with or without TGF- β . In a third setup, monocytes were solitarily cultured in RPMI/10% fetal calf serum (FCS) and likewise supplemented with or without TGF- β . Cell cultures were left untreated, or TNBS or lipopolysaccharide (LPS) was added. After 48 h, upregulation of CD86 was determined as described in Fig. 4. Given are mean-values ($\pm$ SD) of four independent experiments (two different blood and two different skin donors were used).

culture media. Used were either serum-free KGM medium or RPMI/10% FCS (Fig. 7). Tumor growth factor- β treated cocultures reacted more pronounced to TNBS than to LPS. Cocultures without TGF- β treatment were more sensitive to LPS. The variance of CD86-upregulation was lower in TGF- β treated cocultures as compared with those without TGF- β treatment (Fig. 7). Monocytes reacted only scarcely to both stimuli when cultured solitarily in KGM regardless of having been treated with TGF- β (Fig. 7). When stimulated with LPS, monocytes solitarily cultured in RPMI/10% FCS with TGF- β treatment upregulated CD86, sixfold and without TGF- β treatment nearly 19-fold (Fig. 7). In contrast to LPS, TNBS at 100 μ M could not induce a CD86-upregulation in solitarily cultured monocytes, if RPMI/10% FCS was used as culture medium. That applied regardless of addition of TGF- β .

LPS but not TNBS induced secretion of IL-6, TNF- α , and IL-10

Secretion of various cytokines into the culture media was measured by ELISA (not shown). Appreciable levels of IL-6 could be found after addition of LPS only. In all treatment groups (co- or singleculture with or without

addition of TGF- β), the LPS-induced IL-6 concentration was around 500 pg/ml. Results concerning tumor necrosis factor- α (TNF- α) secretion were similar. Again, only LPS induced appreciable levels of TNF- α , but TGF- β treatment downregulated the final amounts. Tumor growth factor- β treated single- and cocultures reached LPS-induced TNF- α levels around 100 pg/ml. Omitting TGF- β yielded a TNF- α concentration of 350 pg/ml in cocultures and of 700 pg/ml in single-cultures. Like TNF- α , IL-10 was secreted after LPS addition only and was dependent on TGF- β treatment. However, IL-10 was not secreted at all by TGF- β treated cell cultures. Cocultures without TGF- β reached LPS-induced IL-10 levels of 100 pg/ml, and single-cultures generated without TGF- β yielded 600 pg/ml. In the media of KC/DC-related cell cocultures, IL-1 β was in the range of 20 pg/ml and did not increase after addition of 100 μ M TNBS (not shown). In conclusion, the TGF- β treated coculture system did not increase the secretion of neither IL-6, TNF- α , IL-10 nor IL-1 β after addition of TNBS.

Discussion

The test kit uses primary human cells which *in vivo* participate in sensitization to contact allergens. On its own, this would not be new. The innovation concerns the constitution of the coculture. Assuming that Langerhans cells may not be continuously attached to epidermal KC, we emulated a putative *in vivo* situation by setting up a loose-fit coculture of KC and monocytes. We attempted to differentiate the cocultured monocytes towards Langerhans cells by culturing in presence of IL-4, GM-CSF, and TGF- β (22). Inclusion of TGF- β not only drives generation towards a Langerhans cell-like type but also reduces the tendency to spontaneous maturation (23). As serum should be omitted from biological assay systems, we chose KGM as the common culture medium for both cell types. Within 6 days of coculture, monocytes in the R2-gate (Fig. 1) changed cellular morphology and phenotype. Cells increased in size, gained distinctive dendrites and lost the typical monocyte surface antigen CD14. Unlike monocytes cultured in serum-containing medium (Fig. 1C), they did not gain expression of Langerin and of CD1a, and achieved only a low level of CD1c (Fig. 1A,B). Monocytes cocultured for 6 days in cytokine supplemented KGM did not resemble Langerhans-like cells or dendritic cell-like cells and were therefore called DC-related cells.

Cocultured KC were activated by the cytokine treatment (Fig. 1D). For example, activated KC secrete elevated amounts of MMP-9 during wound healing (33). Keratinocytes are known to secrete a rich variety of cytokines (reviewed in Ref. 34). In this coculture system, these were combined with the exogenously applied cytokines. Such culture conditions allowed the development of DC-related cells which were sensitive to

contact allergens. So far as analyzed, surface antigen pattern of monocytes/DC-related cells cultured in KGM with the cytokine cocktail were similar with or without cocultured KC (Fig. 1A,B). However, the presence of KC during coculture enabled DC-related cells to sense contact allergens (Fig. 7). In serum-free cocultures supplemented with a cytokine cocktail including TGF- β , CD86-upregulation was induced by TNBS even stronger than by LPS (Fig. 7). Furthermore, inclusion of TGF- β reduced the variance of CD86-upregulation.

In vitro generated Langerhans-like (moLC) and dendritic-like cells (moDC) are known to respond to stimulation to Toll-like receptor 4 ligand LPS by upregulation of CD86 (10, 35) (Fig. 7). However, the experiments were performed using media supplemented with serum. Cytokine-treated monocytes generated under serum-free conditions reacted very scarcely to LPS, but CD86-upregulation in response to LPS occurred when these cells were cocultured with KC (Fig. 7). Regarding detection of LPS, cocultured KC seemed to substitute partially for serum. Regarding detection of 100 μ M TNBS, cocultured KC were more than a substitute for serum. They were absolutely indispensable for sensing the allergen (Fig. 7). Trinitrobenzenesulfonic acid is not a prohaptens which must be metabolized to an immunogenic substance. But proper haptens still need proteinization. This might be a part of the functions provided by KC. It was proposed that KC participate in allergen-induced maturation of dendritic cells by providing danger signals (25). However, the typical danger signal LPS but not TNBS induced secretion of IL-6 and TNF- α into the medium of cocultures (not shown). Interleukin-6 and TNF- α are typical responses of danger signal ligation to Toll-like receptors (review in Ref. 36). So the allergen TNBS is likely to induce maturation of DC-related cells through a route distinct from Toll-like receptor signalling.

Allergen-induced DC-related cell activation could be determined by upregulation of CD86. Upregulation of HLA-DR levels was found to be much less pronounced (not shown). An alternative read-out parameter for future refinement of the assay might be induction of tyrosine phosphorylation (37). Significant CD86-upregulation was shown for the known strong allergens TNBS (Figs 2 and 3), PPD (Fig. 4), and 2-amino-*p*-cresol (Fig. 5). Increase in CD86-MFI occurred proportional to allergen concentrations until toxic levels were reached as detectable by 7-AAD staining. For PPD and 2-amino-*p*-cresol, the toxic limit was low. Sensitizing potential could be detected because DC-related cells in coculture showed an unprecedented sensibility to contact allergens. For example, half-maximal CD86-upregulation occurred in solitarily cultured dendritic cells at more than sixfold higher concentrations of TNBS (21). 4-aminoacetanilide did not reveal significant effects in common LLNA sensitization protocols. Only the use of a modified protocol with a high-concentration challenge resulted in a significant increase of lymphnode weight and cellularity

(32). In concordance, the *in vitro* assay characterized 4-aminoacetanilide as a weak allergen (Fig. 5).

In each assay, 100 μ M TNBS was included as a positive control for functionality. The variance of CD86-upregulation was very low. Even the use of different cell donors did not particularly increase variance (Figs 2 and 3). Such a stable reaction proved the reliability of this assay. For example, the finding that SDS at 20 μ M did not cause a sensitization, is based on experiments in which the same batch of cocultured DC-related cells proved positive for TNBS (Fig. 6). Keratinocyte and monocytes do not need to originate from the same donor as cells of the adaptive immune system are not functionally integrated. However, the presence of some residual lymphocytes, as in case of adherence-based monocyte enrichment, did not influence test results (Fig. 2C). The proposed *in vitro* assay for detection of contact allergens showed a high sensitivity and reproducibility. Comparison of half-maximal effect levels would allow classification of allergens (as strong–

moderate–weak). Individual cell donors did not critically influence test results. The test kit can be set up from cryopreserved cells, and cells were cultured under serum-free conditions. The assay is easy to perform. We would like to invite interested laboratories to participate in further evaluation.

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Conflict of interest

D.Briechele from TeSens has filed a patent for production of the *in vitro* assay and for its use in prediction of sensitizing potential. The other authors state no conflict of interest.

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