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A new dendritic cell type suitable as sentinel of contact allergens

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ABSTRACT

Establishing of alternatives to animal tests is ethically desirable and gains in importance in context of new European Union regulations such as REACH. We have refined our new in vitro assay for prediction of the sensitizing potency of xenobiotics. Monocytes cocultured with primary human keratinocytes develop to a novel class of in vitro generated dendritic cells after treatment with transforming growth factor beta and Interleukin-4 in serum-free medium. These dendritic cell-related cells (DCrc) are the key players in the loose-fit coculture-based sensitization assay (LCSA). Assay duration and cytokine consumption could be cut down without impairing the assay's functionality. DCrc showed a dose-dependent upregulation of CD86 after treatment with the contact allergens 2,4,6-trinitrobenzenesulfonic acid, the prohaptens isoeugenol, and alpha-hexyl cinnamic aldehyde. The metal allergens nickel and cobalt could be detected by measuring Interleukin-6 and macrophage inflammatory protein 1-beta (MIP-1beta, CCL-4) in coculture supernatants. The irritant zinc elicited no reaction. Lipopolysaccharide produced upregulation of CD86, IL-6 and MIP-1beta. Determination of tolerable concentrations of an allergen in consumer products requires a widely accepted sharp quantitative assay. Animal-based assays do not meet this requirement. The LCSA provides dose-response information, thereby allowing prediction of the relative ability of a substance to induce sensitization.

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1. Introduction

As of 2009, the Seventh Amendment to the European Cosmetic Directive restricts animal testing for safety assessment of products and ingredients. However, for the prediction of skin sensitization potential of contact allergy provoking substances, there is currently no validated and legally acceptable alternative method available.

Allergy development is controlled by dendritic cells (DC). DC are professional antigen-presenting cells. For the induction of an immune response, they sense potentially harmful environmental substances by pattern recognition receptors. Contact with these

“danger signals” is then translated and integrated into signaling pathways inducing DC-maturation. Concurrently, antigens are sampled and processed for presentation to T-lymphocytes (for reviews: Steinman and Banchereau, 2007; Medzhitov, 2007). It was speculated that an allergen itself stimulates DC-maturation (Ryan et al., 2007). This would imply that allergens exhibit both a danger signal and an antigenic entity on the same molecule. However, interactions of sensitizing substances with DC are not defined, and crucial properties of an allergen which determine the immune response are unclear.

The function of DC is shaped by communication with their cellular environment. In the skin, keratinocytes may provide danger signal-induced cytokines or chemokines (for review: Vandebriel et al., 2005). For example, DC may be activated by the IL-7 like cytokine thymic stromal lymphopoietin (TSLP) which is preferentially expressed by epithelial cells including keratinocytes in lesions of atopic dermatitis (for review: Liu et al., 2007). An in vitro assay for the prediction of sensitizing potential should therefore contain keratinocytes and DC.

General shortcomings of cell lines (Hartung, 2007; Boonstra et al., 2007) and current unavailability of a cell line that could substitute for real DC (Ryan et al., 2007) intended us to develop an in

Abbreviations: REACH, registration, evaluation and authorisation of chemicals; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HCA, alpha-hexyl cinnamic aldehyde; MIP-1β (CCL-4), macrophage inflammatory protein 1-beta; LCSA, loose-fit coculture-based sensitization assay; DC, dendritic cell; DCrc, dendritic cell-related cell; KGM, keratinocyte growth medium; DMSO, dimethyl sulfoxide; TGF-β, transforming growth factor-β; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide.

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vitro assay for predictive identification of skin sensitization hazards which is based on human primary cells.

Assuming that skin DC may not be continuously attached to epidermal keratinocytes, we emulated a putative *in vivo* situation by setting up a loose-fit coculture of keratinocytes and monocytes. The application of exogenous cytokines induced concomitant keratinocyte activation and differentiation of monocytes to dendritic cells. Within days, the coculture developed to an allergen-sensitive system. Sensitization could be determined by analyzing the expression of the dendritic cell maturation marker CD86. Estimation of the concentration required to cause a half-maximal increase in CD86 expression allowed quantitative risk assessment (QRA) for chemicals. The presence of keratinocytes increased the sensitivity of the DC-related cells to allergens as compared to solely cultured DC (Aeby et al., 2004). This allowed testing at concentration ranges without general toxicity. The new assay was entitled loose-fit coculture-based sensitization assay (LCSA). First encouraging results were reported recently (Schreiner et al., 2007).

Here we report on optimization of LCSA test protocols. We attempted to shorten the assay's run time and to reduce the need for exogenous cytokines. We found that treatment of monocytes with the cytokines TGF- β and IL-4 in the presence of keratinocytes resulted in a new kind of dendritic cells characterized as CD11c⁺, HLA-DR⁺, CD86⁺, CD14[–], CD1a[–], CD1c[–], and Langerin[–]. These cells represent the first dendritic cell type that generates without addition of exogenous GM-CSF. We show that the new dendritic cell-related cells (DCrc) are sensitive sentinels of contact allergens. A read-out system combining analysis of CD86 expression and determination of the cytokine IL-6 and the chemokine MIP-1 β could distinguish between low-molecular-weight contact allergens like the prohaptens isoeugenol and metal allergens and the pathogenic antigen LPS.

2. Materials and methods

2.1. Media and reagents

Cells were cultured in serum-free keratinocyte growth medium-2 (KGM-2; PromoCell, Heidelberg, Germany) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (both: Biochrom, Berlin, Germany). 2,4,6-Trinitrobenzenesulfonic acid (TNBS), nickel chloride (both: Sigma, Munich, Germany), cobalt chloride (Fluka, Munich, Germany), and zinc chloride (Merck, Darmstadt, Germany) were dissolved in sterile pyrogen-free water (DeltaSelect, Dreieich, Germany). Isoeugenol (Fluka) and alpha-hexyl cinnamic aldehyde (HCA, Sigma) were dissolved in dimethyl sulfoxide (DMSO; Hybrimax, Sigma). Final concentrations of DMSO in cell culture media did not exceed 0.2%. Lipopolysaccharide (LPS; *Escherichia coli* serotype 0128:B12) was obtained from Sigma.

2.1.1. Cytokines

Where applicable, IL-4, GM-CSF (both: Immunotools, Friesoythe, Germany) and TGF- β 1 (R&D, Wiesbaden-Nordenstadt, Germany) were added to cell culture media at final concentrations of 100 ng/ml, 100 ng/ml, and 10 ng/ml, respectively.

2.2. 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) for 15 min at 37 °C. Single-cell suspensions were obtained by passing through a 40- μ m cell strainer (BD Biosciences, 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 $(2-5) \times 10^5$ cells/cm². Cells were cultured until confluence with medium changes on alternate days. Finally, keratinocytes were harvested by trypsinization and cryopreserved in fetal calf serum (FCS; Biochrom) with 10% DMSO (Hybrimax, Sigma) at a density of 6×10^6 cells/ml.

2.3. Cryopreservation of human monocytes

Peripheral blood mononuclear cells (PBMC) were enriched from fresh buffy coat preparations (DRK Blutspendedienst, Berlin, Germany) by den-

sity centrifugation on Ficoll-Paque (Biochrom). Monocytes were purified from PBMC by positive selection of CD14⁺ cells using magnetically labeled 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 keratinocytes.

2.4. Loose-fit coculture-based sensitization assay (LCSA)

We refined the previously published LCSA protocol (Schreiner et al., 2007).

Cryopreserved keratinocytes were seeded in 12-well plates (Costar Cell Culture Cluster, Corning) at a density of $(2-6) \times 10^4$ cells/cm² in serum-free KGM-2. When half-confluence was reached, cryopreserved allogenic CD14-isolated monocytes were seeded onto keratinocytes at a density of $(2-5) \times 10^5$ cells/cm². Then, cytokines were added as indicated in Section 3. After generation times ranging from 2 to 6 days, probes were added. After 48 h, non-adherent cells were pipetted off the coculture, counted (CoulterZ1, 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).

2.5. FACS analysis

Antibody staining was measured in a FACS Calibur flow cytometer (BD Biosciences). Uptake of 7-amino-actinomycin (7-AAD; ViaProbe, BD Biosciences) was used to 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), APC anti-human leukocyte antigen-DR (HLA-DR; mouse IgG2a, clone L243, BD Biosciences), PE anti-CD86 (mouse IgG1, clone FUN-1, BD Biosciences), APC anti-CD1a (mouse IgG1, clone HI149, BD Biosciences).

2.6. LCSA-specific FACS analysis

For the LCSA, cells were gated to a distinct population of DCrc in scatter dot plots (Fig. 1A/2A). Additionally, dead cells were excluded from determination of mean fluorescence intensities (MFI) of surface proteins by 7-AAD staining. Absolute values of MFI are dependent on flow cytometer settings. Therefore, we determined relative upregulation of CD86 using the following equation: MFI CD86 in treated cells/MFI CD86 in corresponding vehicle control. Only cocultures with pairs of the same donors during the same experiment were matched.

2.7. ELISA

End point concentrations of IL-6 and MIP-1 β were detected in cell culture supernatants using DuoSet ELISA kits from R&D Systems (Wiesbaden-Nordenstadt, Germany). Results were calculated for 10⁶ harvested cells and corrected in regard to exact volumes of cell culture supernatants.

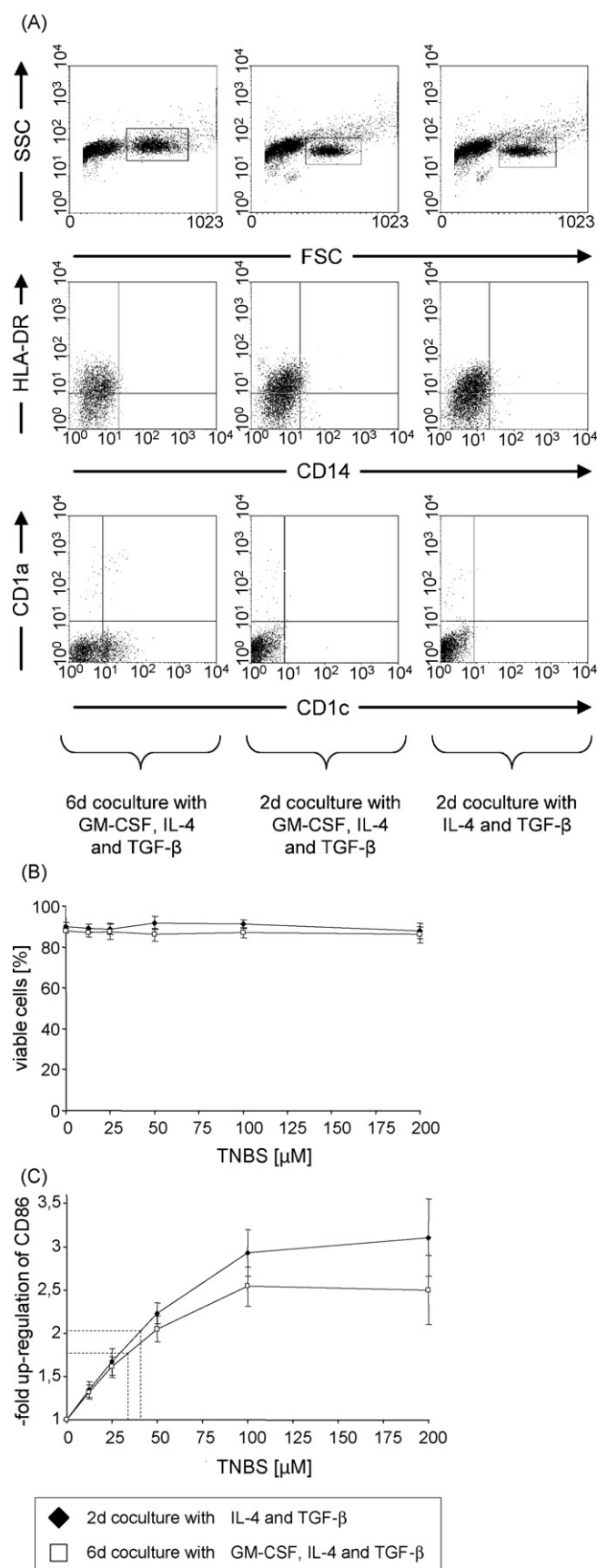
3. Results

3.1. Refinement of the LCSA: reduction of assay duration and cytokine application

Already 48 h after application of GM-CSF, IL-4, and TGF- β , monocytes in coculture with keratinocytes gain light microscopically visible dendrites. Therefore, we questioned whether we could reduce generation time of DCrc from 6 days with a three-time cytokine treatment to 2 days with a one-time cytokine treatment.

We could confirm that DCrc after 2 days and 6 days in coculture with application of GM-CSF, IL-4, and TGF- β show a similar expression of surface antigens (Fig. 1A, left and middle). They lost CD14 almost completely. About half of them were HLA-DR⁺, and they were both CD1a[–]. However, DCrc after 6 days in coculture were CD1c^{dim} and after 2 days CD1c[–]. All cells were CD11c⁺ (not shown).

In a next step, we investigated whether all three cytokines were necessary to generate DCrc from monocytes. We treated cocultures with several combinations of one, two, or three cytokines out of GM-CSF, IL-4, and TGF- β . Also, some cocultures were left untreated. After 2 days, we checked cells for light microscopically visible den-



drates, CD14 expression, and functionally for their response to the strong contact allergen TNBS (Table 1; Fig. 1).

3.1.1. 0 and 1 cytokine

Monocytes in untreated and GM-CSF-treated cocultures showed no forming of dendrites and were mostly CD14⁺. They also failed to react to TNBS (Table 1). Interestingly, monocytes in coculture without exogenous cytokine treatment were almost entirely viable even after 2 days in serum-free medium, as tested by 7-AAD staining (not shown). IL-4 treated monocytes in coculture formed small dendrites but kept their CD14 antigen and showed no reaction to TNBS (Table 1). TGF- β treated monocytes, however, formed larger dendrites, lost CD14 and showed an upregulation of CD86 when treated with TNBS (Table 1).

3.1.2. 2 and 3 cytokines

Monocytes treated once with TGF- β and IL-4 during 2-day coculture with KC showed the same strength of reaction to TNBS as monocytes treated once with TGF- β , IL-4, and GM-CSF (Table 1). Also, 2-day cocultures with one-time application of TGF- β and IL-4 yielded comparable increases in CD86 MFI as 6-day cocultures with three-time application of TGF- β , IL-4, and GM-CSF. Half-maximal upregulation for both settings occurred at 42 μ M and 34 μ M, respectively (Fig. 1B and C). Moreover, GM-CSF application did not alter expression of measured surface antigens (Fig. 1A, middle and right).

We concluded that GM-CSF could be safely omitted from the coculture system. DCrc generation time could be reduced from 6 to 2 days without impairing the functionality of the LCSA (Fig. 1C). The following studies were therefore performed using the improved assay conditions.

3.2. The LCSA can discriminate between TNBS, metal allergens, and LPS by measurement of CD86, IL-6, and MIP-1 β expression

Vehicle control, 100 μ M TNBS as a positive control, 1 μ g/ml LPS, and nickel (Ni), cobalt (Co), and zinc (Zn) at concentrations ranging from 125 μ M to 2000 μ M were applied to LCSA cocultures.

3.2.1. Viability

DCrc treated with vehicle control, TNBS up to 200 μ M, and LPS were mostly viable (Fig. 1B; Fig. 2A and B). Ni-induced cytotoxicity was evident at 500 μ M, at higher concentrations all DCrc were dead (Fig. 2B). Co-induced cytotoxicity was already measurable at 250 μ M with all cells dead at higher concentrations (Fig. 2B). In Zn-treated cocultures DCrc were completely dead even at 250 μ M (Fig. 2A).

3.2.2. CD86 expression

The following data for CD86 expression is only given for conditions where an appreciable part of DCrc survived. Both LPS and TNBS induced a more than twofold higher expression of CD86 in

Fig. 1. Refinement of the LCSA: variations in generation time and cytokine application. CD14-isolated monocytes were cocultured with keratinocytes for 6 or 2 days in the presence of cytokines as indicated. A distinct population of DCrc was identified in scatter dot blots and analyzed for cell surface antigens (A). Shown are results typical of four experiments using four different monocyte and skin donors. TNBS at concentrations ranging from 12.5 μ M to 200 μ M was applied to two different LCSA conditions: 2-day coculture with one-time application of IL-4 and TGF- β (◆); 6-day coculture with three-time application of GM-CSF, IL-4, and TGF- β on alternate days (□). Viability (B, given as % of 7-AAD-negative cells) and upregulation of CD86 (C) were determined. Half-maximal upregulation of CD86 occurred at 34 μ M (◆) and 42 μ M (□). Given are mean values ($\pm$ S.D.) of four independent experiments in duplicates using four different blood and skin donors.

Table 1
Refinement of the LCSA: variations in cytokine application during 2-day cocultures

Cytokines added during 2-day cocultures	Dendrites in light microscopy	CD14 ⁺ cells (%)	-fold change in CD86-MFI after addition of 100 μ M TNBS
None	–	79	0,99
GM-CSF	–	86	0,95
IL-4	+	86	1,07
TGF- β	++	12	1,99
TGF- β , GM-CSF	++	9	1,99
IL-4, GM-CSF	+	81	0,88
TGF- β , IL-4	++	5	2,86
TGF- β , IL-4, and GM-CSF	++	7	3,10

CD14-isolated monocytes were cocultured with keratinocytes in the presence of cytokines as indicated for 2 days. Monocytes were checked light microscopically for morphological changes: –, no dendrites visible in all cells; +, small dendrites visible in a fraction of cells; ++, large dendrites visible in a fraction of cells. Differentiation to DCrc was determined by measuring loss of CD14 and capacity to upregulate CD86 in response to TNBS. Given are combined results of one coherent experiment typical of four experiments with four different blood and skin donors.

DCrc (Fig. 2C). The metal allergens Ni and Co, however, could not increase CD86 levels significantly but Ni showed a statistical trend ($p = 0.057$) towards an CD86 upregulation at 500 μ M. CD86 levels at concentrations higher than 500 μ M could not be measured due to general cytotoxicity.

3.2.3. IL-6 and MIP-1 β

Accumulation of the pro-inflammatory cytokine IL-6 and the chemokine MIP-1 β (CCL-4) was determined in all treatment groups. LPS and metal allergens, but not TNBS, showed a strong induction of IL-6 and MIP-1 β (Fig. 2D and E). Cytokine levels were highest after LPS treatment, followed by treatment with Ni. Ni at concentrations between 500 μ M and 2000 μ M induced a more than 15-fold increase in MIP-1 β levels.

Co showed a less pronounced but still significantly higher ($p < 0.01$) secretion of IL-6 and MIP-1 β as compared to the vehicle control. For both Ni and Co, MIP-1 β was elevated at 250 μ M, whereas IL-6 was significantly higher already at 125 μ M (Fig. 2D and E).

The irritant Zn could not induce appreciable secretion of IL-6 and MIP-1 β at concentrations of 250 μ M and 500 μ M (not shown).

In contrast to CD86, cytokine accumulation could be used as a read-out parameter even in conditions that ended up in massive cell death of DCrc.

3.3. The LCSA can detect the prohaptens isoeugenol as a contact allergen

Experiments with the improved LCSA protocol shows a dose-dependent upregulation of CD86 after treatment with various concentrations of isoeugenol (Fig. 3B). General cytotoxicity occurred at concentrations higher than 500 μ M (Fig. 3A), whereas significantly higher CD86 expression could already be measured at 100 μ M. Half-maximal upregulation of CD86 occurred at around 140 μ M (Fig. 3B).

IL-6 and MIP-1 β secretion was not elevated. For all conditions, MIP-1 β and IL-6 concentrations in cell culture media were around 40 pg/ml and 10 pg/ml, respectively (not shown).

3.4. The LCSA can detect the model allergen alpha-Hexyl cinnamic aldehyde (HCA)

Surprisingly, HCA showed a strong upregulation of CD86 in the LCSA at very low concentrations. Half-maximal upregulation already occurred at 3 μ M (Fig. 4B). General cytotoxicity was not appreciable at concentrations up to 37 μ M (Fig. 4A). At 370 μ M, however, 75% of DCrc were dead (not shown). Secretion of IL-6 and MIP-1 β was not found to be significantly elevated in response to HCA (not shown).

4. Discussion

New product ingredients that may contact the skin must be assessed for their sensitizing potential. Predictive testing is conducted in guinea pigs and increasingly in the murine local lymph node assay (LLNA). There is a need to develop a non-animal test method for skin sensitization. Such an alternative method must prove to be relevant and reliable for prediction of human risks, and it should provide a dose–response relationship allowing relative hazard categorization. We propose the use of our new in vitro assay LCSA. The design of this assay is based on current immunological knowledge of pathogenesis of allergic contact dermatitis. It integrates dendritic cells and keratinocytes which are both known to be critically involved in vivo. The read-out system uses molecular responses typically occurring after encounter of the skin with contact allergens.

The application of exogenous cytokines to the loose-fit coculture of keratinocytes and monocytes induced differentiation of monocytes to a new kind of in vitro generated dendritic cells—dendritic cell-related cells. With a one-time cytokine treatment, they emerge during a coculture time of only 2 days. Typical proteins of established monocyte-derived DC (moDC) like CD1a and CD1c are missing on DCrc (Fig. 1a). Also, Langerin is lacking which is expressed on Langerhans cells and which shows up on dendritic cells when generated from monocytes in the presence of TGF- β (not shown; Schreiner et al., 2007). In conclusion, DCrc can be characterized as CD11c⁺, HLA-DR⁺, CD86⁺, CD14[–], CD1a[–], CD1c[–], and Langerin[–]. To the best of our knowledge, this cell type was not yet described.

DCrc revealed an increased sensitivity to contact allergens as compared to moDC (Aeby et al., 2004). Sensitization could be determined by analyzing upregulation of CD86 and secretion of the cytokine IL-6 and the chemokine MIP-1 β , all of which are typical of dendritic cell activation. Estimation of the concentration required to cause half-maximal increase in CD86 expression allowed quantitative risk assessment for chemicals. However, the molecular basis of the special suitability of DCrc for sensitization testing is currently unknown.

The data provided show that the LCSA test protocol could be refined in terms of assay duration and cytokine consumption making the LCSA both quicker and more economical (Fig. 1; Table 1). As shown in Table 1, GM-CSF never induced significant changes in the outcome of measured parameters. Keratinocytes secrete endogenous GM-CSF into the cell culture medium. However, the interactions of keratinocytes and monocytes/DCrc are complex, and compensation of exogenous GM-CSF by keratinocyte-derived GM-CSF may only partly explain why its addition can be omitted. Surprisingly, the central exogenous cytokine for DCrc-generation was TGF- β (Table 1).

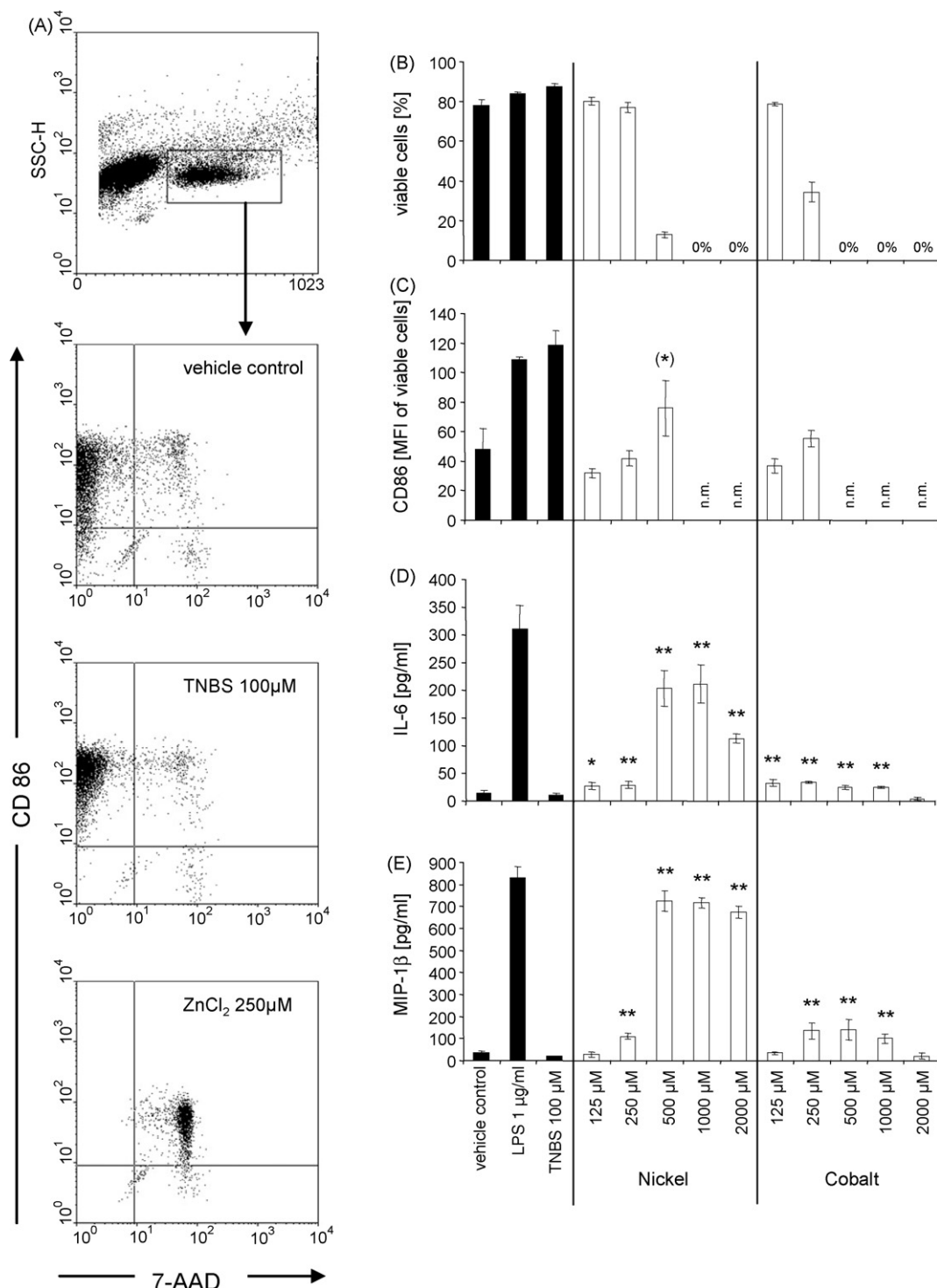


Fig. 2. Differential response of cocultured DCrc to the pathogenic antigen LPS, to the contact allergen TNBS, and to metal salts. CD14-isolated monocytes were cocultured with keratinocytes in the presence of IL-4 and TGF-β for 2 days. Then, LPS, TNBS, NiCl₂, CoCl₂, and ZnCl₂ were added at concentrations as indicated. After another 48 h, cell culture media with non-adherent cells was harvested. For FACS analysis of 7AAD-uptake (B) and CD86 expression (C), cells were gated to the region shown in scatter analysis (A). Lower left quadrants correspond to isotype controls (A; [dot plots show selected conditions only]). The concentrations of IL-6 (D) and of MIP-1β (E) in cell culture media were determined by ELISA. Shown are typical results (mean values ± S.D. of two independent experiments in duplicates using two different blood and skin donors, representative of four independent experiments). (*) $p = 0.057$; * $p < 0.05$; ** $p < 0.01$; n.m., not measurable.

We could improve the reliability of test results by implementing MIP-1β (CCL-4) as additional read-out parameter (Proost et al., 1996). This inflammatory chemokine is connected with T-cell trafficking into lymph nodes (Tedla et al., 1998), and it is associated with a type 1 immune response (Schrum et al., 1996). MIP-1β

guides naïve CD8⁺ T cells to sites of CD4⁺ T cell–dendritic cell interaction and promotes memory CD8⁺ T-cell generation (Castellino et al., 2006). MIP-1β has already been proposed as a biomarker of sensitizing potential (Hirota and Moro, 2006). Using the cell line THP-1, it was shown that secretion of MIP-1β was elevated after treatment

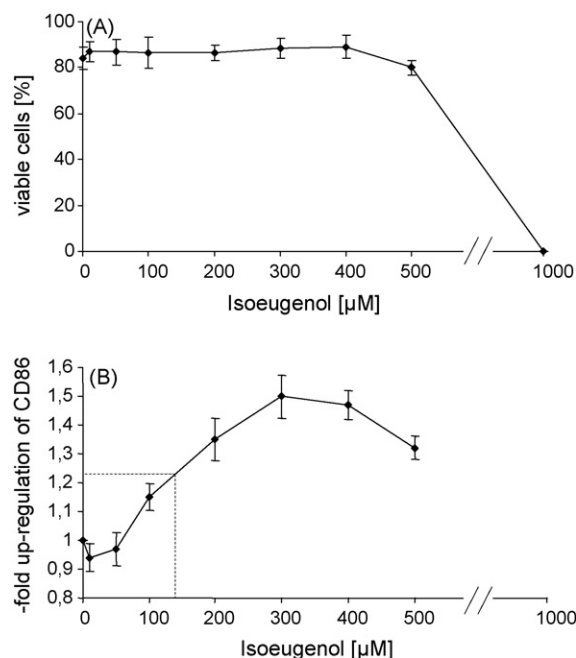


Fig. 3. Isoeugenol-induced cell-death and maturation of DCrc. CD14-isolated monocytes were cocultured with keratinocytes in the presence of IL-4 and TGF- β for 2 days. Then, isoeugenol was added at concentrations as indicated. After another 48 h, viability (A, given as % of 7-AAD-negative cells) and upregulation of CD86 (B) were analyzed. Half-maximal upregulation of CD86 occurred at 140 μ M. Given are mean values ($\pm$ S.D.) of four independent experiments in duplicates using two different blood and skin donors.

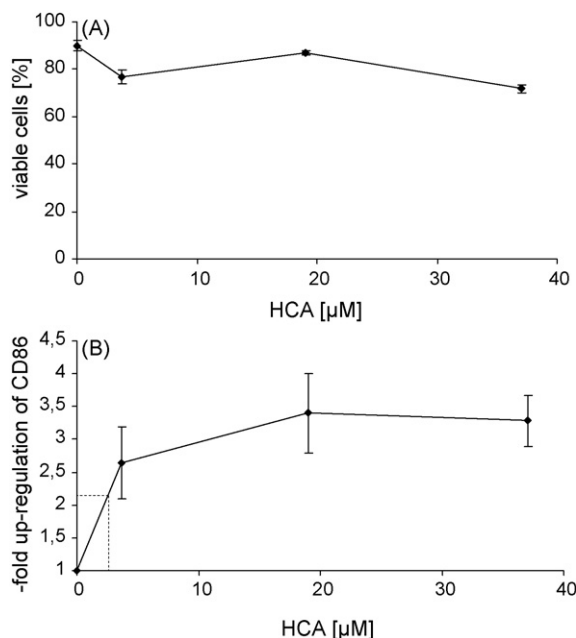


Fig. 4. Alpha-hexyl cinnamic aldehyde (HCA)-induced cell-death and maturation of DCrc. CD14-isolated monocytes were cocultured with keratinocytes in the presence of IL-4 and TGF- β for 2 days. Then, HCA was added at concentrations as indicated. After another 48 h, viability (A, given as % of 7-AAD-negative cells) and upregulation of CD86 (B) were analyzed. Half-maximal upregulation of CD86 occurred at 3 μ M. Given are mean values ($\pm$ S.D.) of four independent experiments in duplicates using two different blood and skin donors.

with the metal allergens nickel and cobalt, whereas application of low-molecular-weight allergens like DNCB and PPD resulted in less pronounced MIP-1 β secretion or even failed to do so (Hirota and Moro, 2006). These results were confirmed in a recent study (Lim et al., 2008). The LCSA yielded comparable results. Nickel and cobalt induced a highly significant increase in MIP-1 β secretion whereas TNBS and isoeugenol did not (Fig. 2). On the other hand, expression of the biomarker CD86 was elevated after application of all of the low-molecular-weight allergens tested but not after application of nickel and cobalt. Only the bacterial antigen LPS elicited an increase in both of these biomarkers (Fig. 2). In the LLNA, nickel yields positive test results only when applied in combination with a vehicle like DMSO or ethanol or in combination with an adjuvant (Ikarashi et al., 1992, 1993). The clinical phenotypes of metal and low-molecular-weight allergens are alike. It remains unclear why in vitro they elicit such a diverse performance regarding MIP-1 β and CD86. However, if used in combination, CD86 and MIP-1 β provide a reliable read-out system. Inclusion of the inflammatory cytokine IL-6 might be considered as a further assay-parameter in the LCSA (Fig. 2).

A new in vitro assay for sensitization should possess the appropriate metabolic competence for the conversion of a prohaptent to a protein-reactive haptent. The fragrance isoeugenol forms such a prohaptent (Bertrand et al., 1997), and the LCSA clearly reacted to it with upregulation of CD86 (Fig. 3). Half-maximal increase was elicited at a concentration of 140 μ M. The strong allergens TNBS and PPD (Brandowski's base) yielded half-maximal CD86 upregulations at 45 μ M (Fig. 1) and 20 μ M (Schreiner et al., 2007), respectively. In LLNA, isoeugenol was categorized as a moderate allergen (Gerberick et al., 2004). Assessment by LCSA arrived at the same conclusion.

The next critical allergen included in this study was HCA, which serves as a positive control substance in OECD's guideline for skin sensitization tests (Steiling et al., 2001; Dearman et al., 2001). Assessment of the sensitizing potential of HCA yielded highly varying results when different assay types were used. The guinea pig maximization test classified HCA as a strong allergen (Arimura et al., 1998). In LLNA, HCA was ranked moderate (for review: Smith Pease, 2003). In vitro, HCA reacted strongly in an assay using the cell line Pam-212 (Arimura et al., 1998) but did not elicit any response when U937 cells were used (Python et al., 2007). Clinical data are missing nearly completely. In the LCSA, HCA was assessed as a strong allergen (Fig. 4).

The aldehyde HCA is a ready haptenic molecule which can react directly with protein. However, the availability of HCA to Langerhans cells after application to the skin surface is influenced by the extent of compound adsorption and by an extensive skin metabolism which may result in further activation or in detoxification (Smith Pease, 2003). HCA is more directly available to DCrc in the LCSA as compared to Langerhans cells in the skin. Compound adsorption and penetration rates have no impact on the LCSA. Therefore, the LCSA should be complemented by an assay that analyses compound availability using a human skin model (Schäfer-Korting et al., 2006). A proper prediction model for assessment of sensitizing potential should then integrate data on dendritic cell maturation and on skin penetration.

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Conflict of interest: A patent for setup and use of the LCSA and its refined version is filed by M. Schreiner, D. Briechele and R. Wanner. All other authors state no conflict of interest.

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