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Cross talk between keratinocytes and dendritic cells: Impact on the prediction of sensitization

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short title: activation of antigen presenting cells

Abstract

Understanding the mechanistic aspects involved in sensitization by chemicals will help to develop relevant preventive strategies. Many potential sensitizers are not directly immunogenic but require activation outside or inside the skin by non-enzymatic oxidation (prehaptens) or metabolic transformation (prohaptens) prior being able to induce an immune response. This necessary activation step has not yet been actively integrated into a cell line-based prediction approach. We cocultured HaCaT keratinocytes with THP-1 as dendritic cell-like cells allowing intercellular interactions. The sensitizing potential was determined by analyzing differences in the expression of CD86, CD40 and CD54 on cocultured THP-1 cells. This new assay setup allowed 1) to distinguish irritants from allergens without influencing cell viability and 2) to discriminate pre/prohaptens from haptens. Under coculture conditions, the prohaptens eugenol, 2-methoxy-4-methylphenol and benzo[a]pyrene induced a significantly higher upregulation of CD86 expression on THP-1. In agreement with the hapten concept, responses to 2,4-dinitrochlorobenzene, Bandrowski's base and the prehapten isoeugenol were not significantly modified. Inhibition of cytochrome P450 or NAD(P)H:quinone oxidoreductase (NQO1) activity reduced the prohaptens-mediated upregulation of CD86 on cocultured THP-1 cells. This coculture assay allowing cross talk between HaCaT and THP-1 cells appears to be suitable for the detection of prohaptens, is reproducible, easy to perform and avoids donor variations. In addition, this assay is a promising approach to understand the impact of cross talk on the prediction of sensitization and once established may be integrated in a future *in vitro* toolbox to detect potential skin sensitizers and may thus contribute to reduce animal testing.

Keywords: coculture; cross talk; keratinocytes; antigen-presenting cells; metabolism; skin sensitization assay

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Introduction

The prevalence of allergic contact dermatitis is steadily increasing. About 20% of the population in Western Europe are sensitized to one or more compounds from their environment (Zug *et al.*, 2009). Hence, it is important to identify and evaluate the sensitizing potential of new compounds to provide an accurate hazard identification and risk assessment regarding their safe use. It has been proposed that some form of oxidation on the skin surface or in the viable skin prior to reacting with proteins containing nucleophilic amino acids is a prerequisite for many known sensitizers (Dupuis and Benezra, 1982). Depending on the mode of the activation process, these chemicals have been classified as prehaptenes when activation occurs via abiotic/bio-chemical transformation or as prohaptenes when enzymatic conversion is involved (Lepoittevin, 2006).

Resulting reactive products are able to form conjugates with skin proteins. The latter are processed by skin immune cells such as Langerhans cells or dermal dendritic cells and are presented to T cells and eventually stimulate an immune response (for a recent review see (Vocanson *et al.*, 2009)). Keratinocytes are also known to participate in this process (Kim *et al.*, 2009), i.e. they generate the microenvironment, provide essential danger signals and are considered to be involved in the protein haptenation process e.g. by supplying enzymes necessary for the metabolic activation of prohaptenes as well as detoxification. Thus, for the *in vitro* prediction of the skin sensitization potential of allergens, especially prohaptenes, contribution of keratinocytes may be required to optimally stimulate dendritic cells. Known prohaptenes include chemicals such as geraniol, 2-aminophenol, 1-naphthol, 2-methoxy-4-methylphenol or eugenol (Gerberick *et al.*, 2009).

To our knowledge, no widely accepted *in vitro* test system is available that combines layered keratinocytes with dendritic cells in order to determine the sensitizing potential of chemicals. Efforts have already been made to combine primary keratinocytes with monocyte derived dendritic cells (Schreiner *et al.*, 2007), human reconstructed epidermis with Langerhans cells (Facy *et al.*, 2004; Facy *et al.*, 2005) or MUTZ-3 derived Langerhans-like cells with a three-dimensional full thickness skin model (Laubach *et al.*, 2011), but the capacity of such systems to detect prohaptens has not been investigated yet and they are handicapped by donor variability issues.

This paper describes coculture conditions that provide the context for intercellular interaction between the metabolically competent keratinocyte cell line HaCaT and the monocyte cell line THP-1 which can be used as model for activation of dendritic cells. We specifically describe the increase in the predictive capacity of this coculture system for prohaptens.

Materials and methods

Chemicals and reagents. Benzo[a]pyrene (B[a]P) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Sodium dodecyl sulfate (SDS) and dimethylsulfoxide (DMSO) were purchased from Carl Roth (Karlsruhe, Germany). Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), L-glutamine, antibiotic/antimycotic solution, trypsin-EDTA and phosphate buffered saline (PBS) were obtained from PAA (Cölbe, Germany). Eugenol, isoeugenol, 2,4-dinitrochlorobenzene (DNCB), resorufin, ethoxyresorufin, dicumarol, lipopolysaccharide (LPS, *escherichia coli* serotype 055:B5), β -mercaptoethanol, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), propidium iodide, Benzalkonium chloride (BZC, predominantly $C_{12}H_{25}N(CH_3)_2C_7H_7Cl$, but also containing C_{14} and C_{16} homologs, therefore molarity is not calculated), 2-methoxy-4-methylphenol (MMP), 1-aminobenzotriazole (ABT) and fetal bovine serum (FBS) were from Sigma-Aldrich (Taufkirchen, Germany), whereas Bandrowski's base (N,N'-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine, BB) was from ICN Biomedicals (Aurora, Ohio USA).

Culture conditions for HaCaT and THP-1 cells. HaCaT cells were cultured in DMEM plus 10% FCS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution and THP-1 were cultured in RPMI 1640 containing 4 mM L-glutamine, 25 mM HEPES, 50 μ M β -mercaptoethanol, 10% FBS and 1% antibiotic/antimycotic solution (further referred to as "THP-medium").

Coculture conditions for measurement of cell surface markers on THP-1. HaCaT cells (0.7×10^6 cells in 2.5 ml culture medium) were cultivated in 6-well plates for 48h yielding

approx. 2×10^6 cells. Cells were washed twice with THP-medium and THP-1 (1.5×10^6 in a final volume of 3 ml) were added to HaCaT cells and were incubated in the absence and presence of compounds for 24h. DMSO (0.2%) served as solvent control. For inhibition of NAD(P)H:quinone oxidoreductase (NQO1) activity, cells were preincubated with 20 μ M dicumarol for 30 min before the addition of eugenol. THP-1 cells were then collected and their expression of cell surface markers was analyzed via flow cytometry. Cytotoxicity was measured by flow cytometry using propidium iodide (PI). In parallel THP-1 cells (1.5×10^6 in 3 ml) were treated as indicated in the absence of HaCaT cells ("monoculture").

Flow Cytometry Analysis. THP-1 cells were stained with fluorescein isothiocyanate (FITC)- or allophycocyanin (APC)-labeled monoclonal antibodies and isotype-matched controls (all mouse IgG1, purchased from BD Pharmingen, Heidelberg, Germany): anti-CD86 (clone 2331 (FUN-1)), anti-CD40 (clone 5C3) and anti-CD54 (clone HA58). Anti-CD11a (clone HI111) was used for discriminating THP-1 from HaCaT cells. Surface marker expression was measured using a FACSCalibur and analyzed with the Cell Quest ProTM software (BD Biosciences, Heidelberg, Germany). PI-positive cells were excluded from analysis. Relative mean fluorescence intensities (rMFI) were calculated by subtracting the MFI of the corresponding isotype controls.

Determination of cytochrome P450 1 (CYP1) enzyme activity. In order to estimate the influence of the tested chemicals on CYP1 enzyme activity under the assay conditions (HaCaT monoculture, HaCaT after coculture with THP-1) ethoxyresorufin *O*-deethylase (EROD) activity was analyzed as a marker for CYP1 activity as described (Bonifas *et al.*, 2010). For inhibition of CYP activity, cells were preincubated with 1-aminobenzotriazole (ABT) for 1h prior addition of eugenol or benzo(a)pyrene. For confirmation of CYP1

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metabolism, formation of 3-OH-B[a]P, a major CYP1 dependent metabolite, was quantified by LC/MS using 500 µl of cell culture supernatant. To determine the conjugation rate of phase II enzymes, subsamples were incubated with β-glucuronidase/arylsulfatase prior to analysis. Calibration standards were prepared by spiking cell culture medium with definite amounts of 3-OH-B[a]P in the range of 1 – 100 µg/l. The limit of detection for 3-OH-BaP was determined to be 0.5 µg/l.

Statistical analysis. Differences between controls and chemical-treated cells among the same culture condition were determined by using Student’s t-test. A P-value <0.05 was considered to be statistically significant.

Results

Impact of HaCaT cells on basal and lipopolysaccharide-induced cell surface marker expression on THP-1 cells

Initially we evaluated the impact of cross talk between the two cell lines on the THP-1 cell surface expression of CD86, CD40 and CD54 after coculture with HaCaT cells for 24h. Whereas expression of CD86 and CD40 on THP-1 cells was reduced after coculture in comparison to monoculture, CD54 expression was significantly increased after coculture with HaCaT, both based on expression per cell (calculated as relative mean fluorescence intensity, rMFI) and the percentages of CD54 positive cells (see figure 1). In order to clarify whether cell-cell contacts or mediators present in the culture medium induced the observed changes, THP-1 cells were cultured in the absence of HaCaT cells in HaCaT-conditioned medium for 24h. Results indicate (see figure 1A-C) that CD86, CD40 and CD54 levels on THP-1 cells cultivated in conditioned medium were comparable to levels observed under coculture conditions, indicating that the down- or upregulation were induced by mediators released by HaCaT cells into the culture medium. THP-1 cells were then incubated with lipopolysaccharide (LPS) for 24h in the absence and presence of HaCaT cells, the same amount of LPS (10 µg/ml, i.e. 30 µg in total) being applied to approx. 1.5 million cells under monoculture (equals 20 µg LPS per 10⁶ cells) and approx. 3.5 million under coculture condition (equals 8.6 µg LPS per 10⁶ cells). Our results indicate that the cell viability was not influenced by coculture conditions and that the expression of all three markers was significantly increased on THP-1 cells after coculture (figure 2). These results confirm basal and induced cross talk between HaCaT keratinocytes and THP-1 as model for dendritic cells and an improved dynamic range regarding CD86 and CD40 expression.

Influence of coculture with HaCaT cells on the expression of CD86, CD40, CD54 and cell viability after exposure of THP-1 cells to the contact allergens 2,4-dinitrochlorobenzene and Bandrowski's base

THP-1 cells were stimulated with 2,4-dinitrochlorobenzene (DNCB) for 24h in the absence or presence of HaCaTs. Cell viability and expression of CD86, CD40 and CD54 were then determined. When comparing cells exposed to equal molarities of DNCB, a significantly reduced upregulation of CD86, CD40 and CD54 was observed on cocultured THP-1 cells (data not shown); when the dose of DNCB is indicated as μg per 10^6 cells. Similar dose-responses were observed for cell viability (figure 3A) and CD86 expression (figure 3B). Comparable results were also obtained for CD40 and CD54 (data not shown).

For the strong hapten Bandrowski's base (BB) we found an upregulation of CD86 (figure 3C) and CD54 (figure 3D) on THP-1 cells after treatment for 24h in both culture conditions. As for DNCB, responses were not comparable for equal molarities. Considering that $10\ \mu\text{M}$ and $20\ \mu\text{M}$ in monoculture nearly fit $20\ \mu\text{M}$ and $50\ \mu\text{M}$ in coculture when calculated as μg BB per 10^6 cells, again similar dose-responses for cell viability, CD86 and CD54 expression were obtained.

Expression of CD86, CD40 and CD54 on cocultured THP-1 cells exposed to the irritants sodium dodecyl sulfate and benzalkonium chloride

The irritant sodium dodecyl sulfate (SDS) is considered as a non-sensitizing irritant but is known for false positive results in the local lymph node assay (LLNA) (Basketter *et al.*, 1994), in *in vitro* sensitization assays using primary dendritic cells, and related cell lines (summarized in (Dos Santos *et al.*, 2009)). Thus, we investigated SDS in mono- and coculture and did not detect any increase in the expression of CD86, CD40 or CD54 (figure 4) using $50 - 200\ \mu\text{M}$ SDS.

For many chemicals, a certain degree of cytotoxicity is required before increases in CD86 and/or CD54 expression are observed and therefore we chose benzalkonium chloride (BZC) as a highly cytotoxic irritant (Sakaguchi *et al.*, 2009). We evaluated the impact of BZC on mono- and cocultured THP-1 cells using 0.25 - 2 µg/ml BZC, resulting in a decrease (up to 50%) in cell viability. Expression of CD86 and CD40 was not modulated by BZC, whereas CD54 was upregulated in mono- and coculture (1.9-fold and 2.7-fold, respectively). The maximal effect was achieved with concentrations yielding 75 - 80% cell viability.

Impact of coculture on cytochrome P450 1 activity in HaCaT keratinocytes

Cytochrome P450 1 (CYP1) is known to activate numerous contact allergens including Δ^3 -carene (Duisken *et al.*, 2005), geraniol (Hagvall *et al.*, 2008), carvoxime (Ott *et al.*, 2009), eugenol (Basketter *et al.*, 1996; Sieben *et al.*, 2001) and benzo[a]pyrene (B[a]P) (Gehly *et al.*, 1979). In order to demonstrate the metabolic capacity of HaCaT in our assay we investigated the impact of the two prohaptens B[a]P and eugenol on CYP1-mediated ethoxyresorufin-*O*-deethylase (EROD) activity in HaCaT cells. It must be kept in mind that the same concentrations of test compounds were applied to different cell numbers in both culture conditions, yielding a lower dose calculated as µg per 10⁶ cells in coculture compared to monoculture. Results demonstrate that both B[a]P and eugenol significantly induce EROD activity in HaCaT cells and the response was even enhanced after coculture with THP-1 cells (figure 5). To confirm CYP1 metabolism, we analyzed the formation of 3-hydroxylated B[a]P, a classical CYP1-dependent metabolite of B[a]P, after 24h treatment of HaCaT. A concentration-dependent accumulation of 3-OH-B[a]P was detected, approx. 52% of 3-OH-B[a]P (10-30 ng/ml after incubation with 1µM B[a]P) was conjugated with sulfates or glucuronides, demonstrating the activity of phase II enzymes in HaCaT cells.

The coculture system distinguishes between the prehaptens isoeugenol and prohaptens

In order to evaluate whether the presence of HaCaT cells improves the THP-1 response to prohaptens we investigated eugenol, 2-methoxy-4-methylphenol (MMP), and B[a]P. In addition, we evaluated the capacity of our approach to discriminate between pre- and prohaptens. CD86 expression was thus analyzed on THP-1 cells after treatment with isoeugenol, eugenol, MMP and B[a]P in mono- and coculture conditions. Coculture conditions led to a slightly diminished CD86 expression after exposure to isoeugenol (figure 6A), while inducing a significant increase in CD86 expression after exposure to eugenol (for 900 µM eugenol, upregulation of CD86 compared to DMSO-treated cells increased from 1.4-fold in monoculture to 1.9-fold in coculture, figure 6B) and an improved dose-response relationship for MMP (figure 6C). In case of B[a]P upregulation of CD86 was only detected on cocultured THP-1 cells after incubation for 48h (figure 7A).

Impact of cytochrome P450 1 as well as NAD(P)H:quinone oxidoreductase activity on upregulation of CD86 on cocultured THP-1 cells

Finally, we investigated whether the increased responsiveness of cocultured THP-1 cells to eugenol and B[a]P is related to xenobiotic metabolism. In preliminary experiments we found a dose dependent inhibition of EROD activity by 1-aminobenzotriazole (ABT), yielding a residual activity of approx. 10 pmol/min/mg protein at 2 mM after treatment of HaCaTs with 1 µM B[a]P. Higher concentrations of ABT could not be applied due to unspecific cytotoxicity. Interestingly, 2 mM ABT was not able to influence eugenol induced EROD activity or CD86 upregulation (data not shown). In contrast, ABT inhibited B[a]P-induced EROD activity in HaCaT cells (by 77.5% and 89.2% for 1 and 5 µM B[a]P, respectively) as well as upregulation of CD86 on THP-1 cells (figure 7A) after 48h incubation. Control

experiments demonstrated that incubation with ABT did not generally interfere with upregulation of CD86 (data not shown). Next, we tested dicumarol as an inhibitor of NAD(P)H:quinone oxidoreductase (NQO1) activity, an enzyme highly expressed in skin cells. Upregulation of CD86 on cocultured THP-1 cells after treatment with eugenol (24h) was clearly reduced by dicumarol (i.e. from 33.0 rMFI to 22.8 rMFI for 900 μ M eugenol), whereas the background expression as well as the response to DNCB was not altered (figure 7C).

Discussion

Many compounds are not directly immunogenic but are activated on the surface or inside the skin by non-enzymatic oxidation (prehaptens) or metabolic transformation, e.g. cytochrome P450 (prohaptens) prior to the induction of an immune response. In addition, intercellular interactions between the innate and adaptive immune system after encounter with chemicals are gaining much attention. For instance toll-like receptor (TLR) signaling has been shown to be involved in the sensitization process to 2,4,6-trinitro-1-chlorobenzene (Martin *et al.*, 2008) and 2,4-dinitrofluorobenzene (Akiba *et al.*, 2004) in mice.

The suitability of THP-1 as model for dendritic cells for the *in vitro* prediction of sensitizers has repeatedly been demonstrated by Sakaguchi and co-workers among others (Sakaguchi *et al.*, 2010). A recent study showed a reinforced activation of monocyte derived dendritic cells due to the danger signal adenosine 5'-triphosphate (ATP) secreted by TGF- β 1-treated HaCaT cells (Ohtani *et al.*, 2009), demonstrating the promoting role of a cross talk with HaCaTs (as a model for epithelial cells). This supports the notion that intercellular interactions with HaCaT cells may increase the activation of THP-1 cells in response to sensitizers. However, most dendritic cell-based *in vitro* test systems comprise only one cell type and lack cross talk with keratinocytes. Here we propose a coculture system that provides a direct contact between HaCaT keratinocytes and the monocytic THP-1 cells.

To evaluate if the increased sensitivity obtained with the THP-1 / HaCaT coculture after exposure to LPS was detrimental to the specificity, we tested sodium dodecyl sulfate (SDS) since this irritant is known to induce false positive results in several assays including the local lymph node assay (LLNA) (Basketter *et al.*, 1994). SDS did not significantly modulate any of the markers chosen for this coculture assay. Similar to others (Sakaguchi *et al.*, 2010), we found that the irritant benzalkonium chloride (BZC) led to a significant

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3 augmentation of CD54 on THP-1 in monoculture, which was even slightly enhanced in
4 coculture (1.9-fold and 2.7-fold, respectively). In contrast, expression of the costimulatory
5 molecules CD86 and CD40 was not modified. This emphasizes the need for careful definition
6 of thresholds for these cell surface molecules, pointing to a higher threshold for CD54
7 compared to CD86. This is in line with the findings of Sakaguchi and co-workers for
8 monocultured THP-1 cells (Sakaguchi *et al.*, 2009). Thus, coculturing THP-1 and HaCaT
9 cells may generate a microenvironment that fosters THP-1 activation without decreasing the
10 capacity of the test system to discriminate between sensitizers and irritants. This finding is
11 supported by several studies demonstrating a differentiated response of HaCaT cells to
12 sensitizers or irritants/non-sensitizers (Emter *et al.*, 2010; Vandebriel *et al.*, 2010).

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15 Phase I enzymes, in particular cytochrome P450 1 (CYP1), are known to activate
16 several compounds such as geraniol (Hagvall, et al., 2008), carvoxime (Ott, et al., 2009),
17 eugenol (Basketter *et al.*, 1996; Sieben *et al.*, 2001), and B[a]P (Gehly *et al.*, 1979). In this
18 study we found that the proposed coculture system increased CYP1 activity in HaCaT cells
19 after treatment with the prohaptens benzo[a]pyrene and eugenol. On the other side, phase II
20 enzyme activities may deactivate compounds. Thus, we included the hapten 2,4-
21 dinitrochlorobenzene (DNCB) as an example that is known to be conjugated with glutathione
22 via glutathione-S-transferases (GST) (Pickard *et al.*, 2009). Our results indicate that GST
23 capacities of HaCaT (Zhang *et al.*, 2002) and THP-1 cells (Darley-Usmar *et al.*, 1991) do not
24 interfere with the detection of the THP-1 response under coculture conditions nor did
25 coculture result in decreased sensitivity in case of DNCB.

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28 In order to evaluate the capacity of the coculture system to distinguish between pre-
29 and prohaptens, we applied the prehaptens isoeugenol to our test system and detected a slightly
30 reduced upregulation of CD86 on THP-1 cells in the presence of HaCaT. This finding is in
31 agreement with the accepted notion that due to its prehaptens nature isoeugenol does not need

metabolic activation for its conversion into a protein-reactive compound (Bertrand *et al.*, 1997; Gerberick, et al., 2009). Furthermore, the slightly reduced response in coculture may be explained by the presence of additional phase II enzymes provided by HaCaT, this latter hypothesis is supported by results indicating that isoeugenol is rapidly conjugated by phase II enzymes (Badger *et al.*, 2002). Moreover, we found that the addition of the classical phase I and II inducer and substrate benzo[a]pyrene (B[a]P) to HaCaT cells resulted in the formation of 3-hydroxylated B[a]P (approx. 48% as unconjugated form), indicating both phase I and phase II metabolic activities in HaCaT cells. Hence a decreased response in the presence of prehapten in our assay may occur and point - at least partly - to detoxification capacities of the coculture system.

In contrast to prehapten, prohapten need enzymatic conversion (e.g. by cytochrome P450 enzymes or peroxidases) for acquiring protein binding capacity. Exposure to the prohapten 2-methoxy-4-methylphenol (MMP) led to an increased, dose-related upregulation of CD86 on cocultured THP-1 compared to monoculture. MMP has been reported to yield low quantities of reactive quinone methide (much higher amounts were detected after exposure to eugenol) after incubation with microsomes (Bolton *et al.*, 1995), whereas a major impact of peroxidases on peptide reactivity has been demonstrated (Gerberick, et al., 2009). This supports our hypothesis that enhanced responsiveness to MMP could rather be due to peroxidase than CYP1 metabolism. For eugenol, another prohapten, we measured a significant increase of CYP1 activity in HaCaT cells as well as a significantly enhanced CD86 response on the THP-1 cells under coculture conditions. Varying results have been published regarding eugenol and CYP1. We and others found a significant increase of CYP1 induction (Al Masaoudi *et al.*, 2001; McKim *et al.*, 2010) and enzyme activity (Rompelberg *et al.*, 1993), whereas Han *et al.* detected a significant inhibition of CYP1 induction when MCF-7 cells were co-treated with eugenol and the polycyclic aromatic hydrocarbon (PAH)

7,12-dimethylbenz[a]anthracene (Han *et al.*, 2007), which might be due to different cell types or the simultaneous treatment with the PAH.

Inhibition experiments using the CYP inhibitor 1-aminobenzotriazole (ABT) showed that ABT inhibits B[a]P-induced CYP1 activities followed by a clear reduction of CD86 upregulation on cocultured THP-1 cells. It is known that enzymatic conversion of eugenol leads to quinones (Bertrand *et al.*, 1997) which can then serve as a substrate for NAD(P)H:quinone oxidoreductase (NQO1) (Stiborova *et al.*, 2003; Stiborova *et al.*, 2002). For many skin sensitizers (Ade *et al.*, 2009; Python *et al.*, 2009), like eugenol (Han, *et al.*, 2007), the capability of inducing NQO1 has been demonstrated. We therefore tested the impact of the NQO1 inhibitor dicumarol (Lubet *et al.*, 1985) on eugenol-induced CD86 upregulation and detected a significantly reduced expression of CD86 on THP-1 cells. These results support our hypothesis that coupling competent keratinocytes with THP-1 cells allows or improves the detection of prohaptens. The main advantages of coculturing are improved metabolism activities and the provision of danger signaling resulting in a broader dynamic range of detection and clearer dose-response curves (see figure 6).

It is commonly accepted that no single assay will be able to replace animal tests for the determination of skin sensitization potential of chemicals (See for example (Aeby *et al.*, 2010)). Instead, an integrated testing strategy or a toolbox consisting of a combination of several *in vitro* assays each reproducing one of the many biological steps necessary for skin sensitization has been proposed (Basketter and Kimber, 2009; Divkovic *et al.*, 2005; Jowsey *et al.*, 2006). Recently, the first *in chemico* predictive assay that integrates enzymatic activation with subsequent peptide binding was proposed (Gerberick, *et al.*, 2009). This combination allowed to distinguish prohaptens from irritants and prehaptens, but with a variable impact on the peptide depletion rate. For two of the seven analyzed prohaptens, the oxidation step increased their peptide reactivity by less than 10%. Other approaches are using

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recombinant P450 enzymes in order to generate immunogenic metabolites (Bergstrom *et al.*, 2007). More recently, Chipinda et al. published a test system using THP-1 cells coincubated with hepatic microsomes for the identification of prohaptens (Chipinda *et al.*, 2011). Although promising, these approaches do not provide a balance between activation and detoxification activities or intercellular communication. First experiments with a limited number of chemicals indicate that the proposed combination of HaCaT and THP-1 cells in a coculture might be an optimization of the test system by including activation as well as detoxification events that are relevant for human skin.

In summary, the THP-1 / HaCaT coculture protocol described here represents a promising model for 1) understanding the impact of cross talk on THP-1 activation and 2) the development of a competent *in vitro* sensitization assay that may be included in a toolbox for reducing animal tests.

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Figure legends:

FIG. 1. Impact of HaCaT cells on the expression of CD86, CD40 and CD54 on the cell surface of THP-1 cells. THP-1 cells were cultured for 24h alone or in the presence of HaCaT cells or in culture medium of untreated HaCaT cells ("conditioned THP-medium"). Expression of the cell surface markers CD86 (A), CD40 (B) and CD54 (C) on viable cells was measured via flow cytometry. Bars represent relative mean fluorescence intensities (rMFI) $\pm$ standard error of the mean (S.E.M.), squares display mean values percentages of CD86, CD40 or CD54 positive cells $\pm$ S.E.M. of at least 4 independent experiments, asterisks indicate significant differences (* p <0.05, ** p <0.01, *** p <0.001); n.s. not significant.

FIG. 2. Surface expression of CD86, CD40 and CD54 on THP-1 cells after treatment with LPS for 24h in the absence and presence of HaCaT cells. THP-1 cells were exposed to LPS or medium alone in the absence or presence of HaCaT cells for 24h. Expression of CD86 (A), CD40 (B) or CD54 (C) on viable cells was determined via flow cytometry. Bars show the mean value of the rMFI $\pm$ S.E.M. of three independent experiments, scatters indicate the mean of viable cells in % $\pm$ S.E.M., and asterisks indicate significant differences from untreated control cells (* p <0.05, ** p <0.01, *** p <0.001).

FIG. 3. Impact of the haptens DNCB and BB on cell viability and expression of CD86 and CD54 on mono- and cocultured THP-1 cells. THP-1 cells were exposed to DNCB (A, B) or BB (C, D) in the absence or presence of HaCaT cells for 24h. (A) Cell viability (percentage of PI-negative cells) and (B) expression of CD86 on viable cells after incubation with DNCB and expression of CD86 (C) and CD54 (D) after treatment with BB were determined via flow cytometry. White dots and bars indicate the values obtained with monocultured THP-1 cells,

black dots and gray bars the values obtained with cocultured THP-1, asterisks indicate significant differences from DMSO-treated control cells (* $p < 0.05$, ** $p < 0.01$). Results from 1 - 9 independent experiments are presented; when multiple values were determined, mean $\pm$ S.E.M. are indicated.

FIG. 4. CD86, CD40 and CD54 analysis (rMFI) on THP-1 cells after exposure to sodium dodecyl sulfate (SDS) and benzalkonium chloride (BZC) for 24h in the absence or presence of HaCaT cells. THP-1 cells were exposed to SDS (A, B) or BZC (C, D) in the absence (A, C) or presence (B, D) of HaCaT cells for 24h. Expression of CD86, CD40 and CD54 on viable cells was determined via flow cytometry. Bars show the mean values of the relative mean fluorescence intensity (rMFI) $\pm$ S.E.M. of 2-4 independent experiments. Black scatters display the mean of viable cells (percentage of PI-negative cells) in % $\pm$ S.E.M., and asterisks indicate significant differences from untreated control cells (** $p < 0.001$).

FIG. 5. Impact of THP-1 coculture on basal and induced EROD activity. Confluent HaCaT cells were incubated with (A) benzo(a)pyrene (B[a]P) or (B) eugenol (Eu) in the absence (white bars) or presence (gray bars) of THP-1 cells. After 24h THP-1 cells were removed and the EROD activity was measured in the remaining intact HaCaT cells. The mean $\pm$ S.E.M. from 3-13 independent experiments are indicated, asterisks indicate significant differences from DMSO-treated control cells (** $p < 0.001$).

FIG. 6. Impact of HaCaT cells on CD86 expression and viability of THP-1 cells after treatment with isoeugenol, eugenol and 2-methoxy-4-methylphenol (MMP). THP-1 cells were exposed to isoeugenol (A), eugenol (B) and MMP (C) in the absence (white bars and scatters) or presence (gray bars and black scatters) of HaCaT cells for 24h. Expression of

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FIG. 7: Impact of the enzyme inhibitors ABT and dicumarol on expression of CD86 on THP-1 cells after incubation with benzo[a]pyrene (B[a]P) and eugenol, respectively. THP-1 cells were incubated with B[a]P in the absence (white bars) or presence (gray bars) of HaCaT cells for 48h; 1-aminobenzotriazole (ABT, patterned bars) was added 1h prior to B[a]P (A). Cocultured cells were preincubated with dicumarol (patterned bars) for 30 min before the addition of eugenol (B). After incubation for 24h, expression of CD86 on viable THP-1 cells was determined via flow cytometry. Bars show the mean values of the relative mean fluorescence intensity (rMFI) $\pm$ S.E.M. of 2-13 independent experiments, scatters display the mean of viable cells (percentage of PI-negative cells) in % $\pm$ S.E.M..

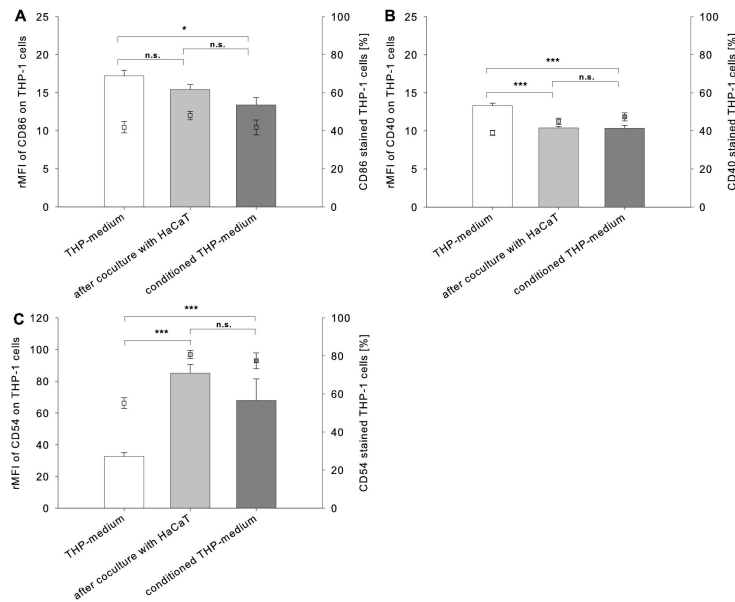


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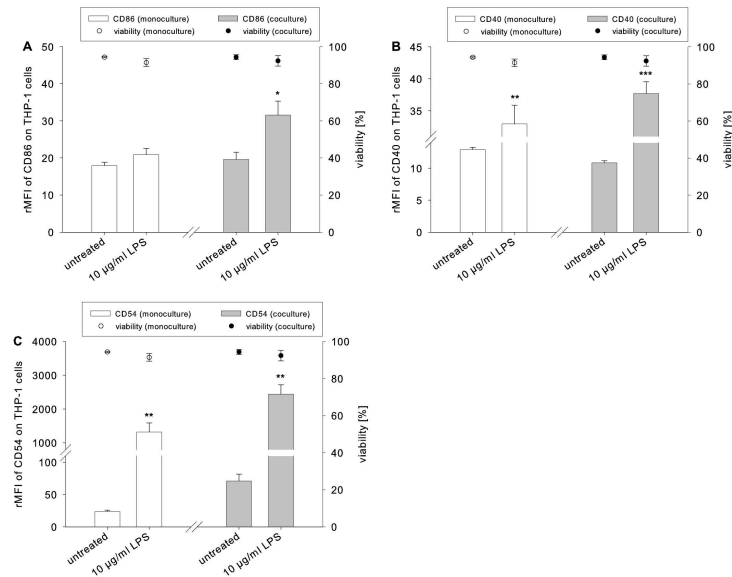


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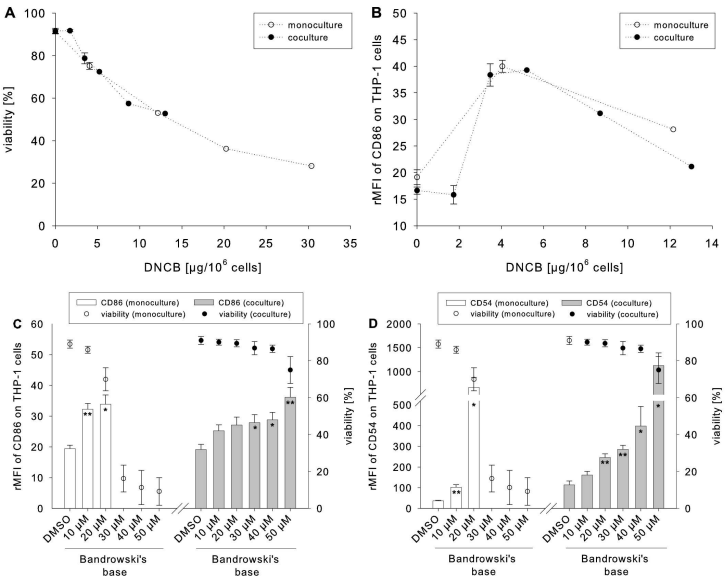


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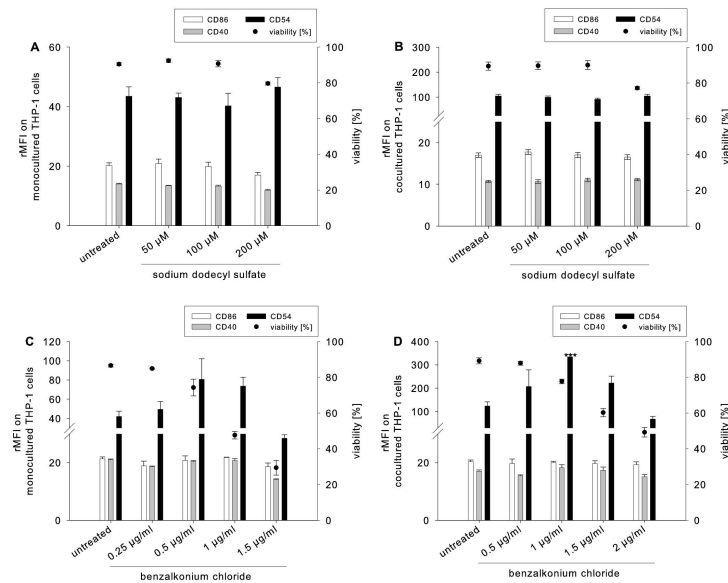


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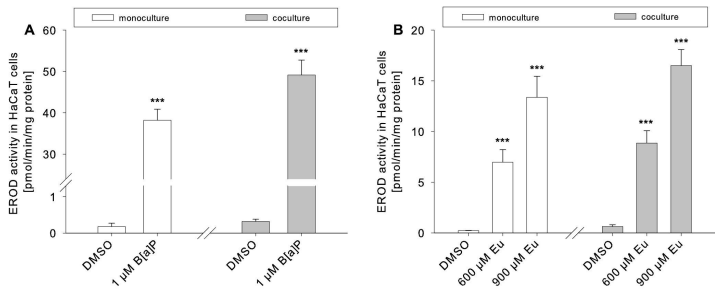


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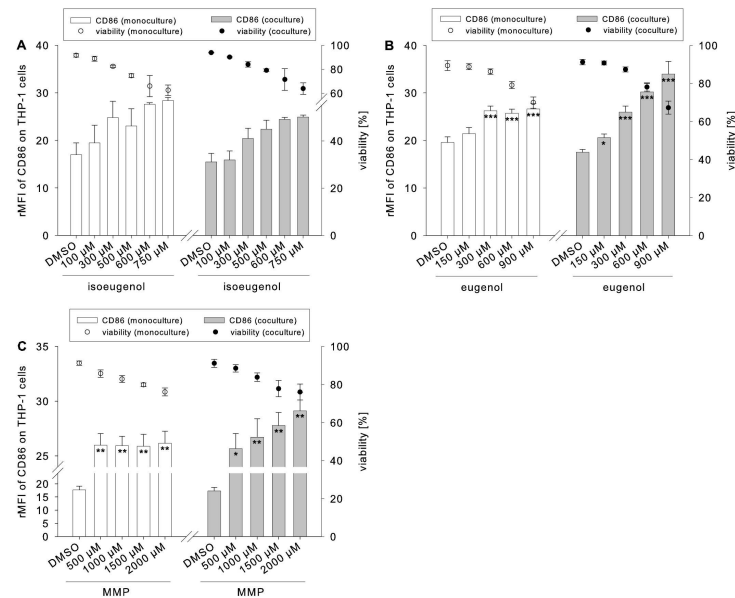


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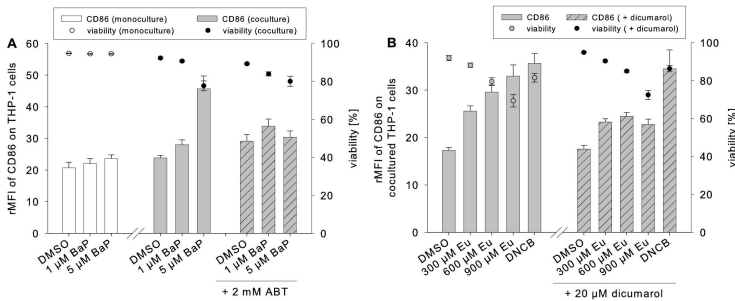


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