

In vitro and *in vivo* analysis of pro- and anti-inflammatory effects of weak and strong contact allergens

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Abstract: Inflammation is a crucial step in the development of allergic contact dermatitis. The primary contact with chemical allergens, called sensitization, and the secondary contact, called elicitation, result in an inflammatory response in the skin. The ability of contact allergens to induce allergic contact dermatitis correlates to a great extent with their inflammatory potential. Therefore, the analysis of the sensitizing potential of a putative contact allergen should include the examination of its ability and potency to cause an inflammation. In this study, we examined the inflammatory potential of different weak contact allergens and of the strong sensitizer 2,4,6-trinitrochlorobenzene (TNCB) *in vitro* and *in vivo* using the contact hypersensitivity model, the mouse

model for allergic contact dermatitis. Cytokine induction was analysed by PCR and ELISA to determine mRNA and protein levels, respectively. Inflammation-dependent recruitment of skin-homing effector T cells was measured in correlation with the other methods. We show that the sensitizing potential of a contact allergen correlates with the strength of the inflammatory response. The different methods used gave similar results. Quantitative cytokine profiling may be used to determine the sensitizing potential of chemicals for hazard identification and risk assessment.

Key words: Arnica – contact dermatitis – inflammation – sesquiterpene lactone

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Introduction

Allergic contact dermatitis (ACD) is an inflammatory skin disease that is caused by small organic chemicals and metal ions (1,2). These haptens must react with proteins, a prerequisite for their recognition by T cells (1,3). The development of ACD proceeds via two different stages. During the sensitization phase, contact allergens penetrate the skin and activate innate immune cells such as epidermal Langerhans cells (LC) and dermal dendritic cells (DC) as well as epithelial and stromal cells, such as keratinocytes. The activation of the innate immune system by contact allergens results in skin inflammation (4,5). Activated DC then leave the skin, upregulate the co-stimulatory molecules CD80, CD86 and CD40 and carry the contact allergens to the skin-draining lymph nodes. There, the contact allergens are presented in

the form of hapten-modified peptides on MHC molecules on the DC to naive T cells. Hapten-specific T cells are then activated by the DC, proliferate and differentiate to effector T cells that enter the blood circulation. An important signal delivered by the skin-derived DC to the T cells induces a skin-specific homing receptor profile that allows effector T cells to infiltrate the skin upon re-exposure of the skin to the contact allergen during the elicitation phase (6,7). The innate inflammatory response then initiates the recruitment of the effector T cells that destroy skin cells such as keratinocytes because of cytotoxic activity and produce inflammatory cytokines such as IFN- γ . Some contact allergens induce Th2 responses. However, that may depend very much on the dose, application protocol and the mouse strain (8–14) as also observed by us for FITC (15).

In every step of CHS development, different cytokines are expressed and required for an efficient sensitization. IL-1 β is mainly produced by LC but also by keratinocytes within 15 min after hapten contact (16). During that phase, IL-1 β mRNA can be detected in the dermis and the epidermis (17). IL-1 β induces the TNF- α production of

Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; DC, dendritic cell; LC, Langerhans cell; TNCB, 2,4,6-trinitrochlorobenzene.

keratinocytes, which in turn serves as a signal for the LC emigration (18). LC and dermal DC transport the contact allergen to the lymph node, and the spectrum of DC-derived cytokines determines Th cell polarization (19). In CHS, Th1/Tc1 differentiation is driven by IL-12 and IFN- γ expression (19). Although CHS represents a Th1/Tc1 response (1), IL-4 is required in an early NKT cell-dependent phase (20,21). When the activated T cells emigrate from the blood into the dermis and epidermis during the elicitation phase, they release pro-inflammatory cytokines like IFN- γ , which in response leads to the increased production of IL-1, IL-6 or IL-8 in keratinocytes (1,22).

Besides the pro-inflammatory cytokines, anti-inflammatory cytokines are involved in the regulation of CHS reactions by regulatory T cells (Treg) (23). IL-10 as one of the most important immunosuppressive cytokines is not only produced by Treg but also by mast cells (24) and regulatory B cells (25) in CHS. One of the effects of IL-10 is the downregulation of the antigen-presenting function of LC for Th1 cells (26).

Because the expression of the mentioned cytokines is very important for the development of CHS, the qualitative and quantitative analysis of the cytokine spectrum may allow the evaluation of the sensitizing potency of putative contact allergens (5,13). In this study, we extend our previous work on the sesquiterpene lactones (SLs) from *Arnica montana*, which are weak contact allergens in the CHS model (27). SLs have anti-inflammatory effects by suppressing NF- κ B activation in human and murine keratinocytes and dendritic cells (DC) as well as DC activation. These effects are dominant *in vivo*. Thus, SLs fail to induce CHS and prevent the induction of CHS in C57BL/6 mice. Pretreatment of the skin with SLs prior to sensitization with TNCB suppresses CHS efficiently. The CHS model may therefore be useful to assess the efficiency of topical drugs for treatment of ACD in the prevention of sensitization and elicitation of CHS (28). Further studies have to demonstrate whether even elicitation of CHS can be reduced by drugs, such as *Arnica* preparations. Only acute depletion of all CD4+ T cells allows the induction of CHS to the more reactive SLs in the Central European (CE) *Arnica* tincture (27). Here, we analysed the expression of the NF- κ B-dependent pro-inflammatory cytokines IL-1 β , IL-6 and of the Th1/Tc1 effector cytokine IFN- γ as well as the Th2 cytokine IL4 and the anti-inflammatory cytokine IL-10 after treatment with weak contact sensitizers, i.e. *Arnica* tinctures containing SLs (27) and with the strong contact sensitizer 2,4,6-trinitrochlorobenzene (TNCB) by quantitative real-time PCR (qRT-PCR) on the gene expression level and by ELISA on the protein level. Afterwards, we compared these results with a previously established *in vivo* assay, the skin-homing assay, which allows to correlate the potency of a contact allergen to

induce skin inflammation with the amount of skin-homing CD8+ effector T cells to inflamed skin (6). Our results demonstrate that the weak allergenicity of SLs in the CHS model correlates with the failure to efficiently induce pro-inflammatory cytokines and contact allergen-induced innate responses that allow the recruitment of skin-homing effector T cells. These findings are concordant with clinical experiences regarding to relative potency of TNCB and SLs to induce ACD (27).

Material and methods

Animals

C57BL/6 (B6) and TCR transgenic P14 mice (29) were provided by the breeding facility of the University Medical Center Freiburg. Female C57BL/6 mice (6–8 weeks old) were purchased from Charles River Laboratories (L'Arbresle, France). All experimental procedures were in accordance with the University Medical Center Freiburg and national guidelines on animal welfare and have been approved.

Test compounds

Arnica tinctures: 'Spanish' *Arnica* tincture was prepared from flowers of *A. montana*, Spanish chemotype (SP), and contained mainly 11 α ,13-dihydrohelenalin esters (0.70 mg/ml calculated as 11 α ,13-dihydrohelenalinmethacrylate) (30). "Central European" *Arnica* tincture (CE) was prepared from *A. montana* flowers, Central European chemotype, by percolation according to the European Pharmacopoeia 1997 and contained mainly helenalin esters (0.83 mg/ml calculated as helenalinisobutyrate) (30). 11 α ,13-Dihydrohelenalinmethacrylate was isolated from flower heads of *A. montana* from the Spanish chemotype and helenalinisobutyrate from the Central European chemotype as described (31). Identity was confirmed by NMR and MS analysis, and purity was evaluated by gas and thin-layer chromatography analyses to be >98% (32).

2,4,6-Trinitrochlorobenzene (TNCB) was from VeZerf Laborsynthesen GmbH, Idar-Oberstein, Germany.

Media, chemicals and antibodies

RP-10 consisted of RPMI 1640 (Gibco, Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine (Gibco), 25 mM HEPES buffer (Gibco), 50 μ g/ml penicillin-streptomycin (Gibco) and 10 μ M 2-mercaptoethanol (Sigma, Deisenhofen, Germany).

Anti-CD8 α (53–67) and anti-TCR V α 2 (B20.1) were from BD PharMingen (Heidelberg, Germany) and were used as FITC, PE or Biotin conjugates. Biotinylated antibodies were revealed by Streptavidin-Cy-Chrome[®] (BD PharMingen).

Primers and probes

The following primers and probes were received from Roche: β -actin: 5'-AAGGCCAACCGTGAAAAGAT-3' (forward), 5'-GTGGTACGACCAGAGGCATAC-3' (reverse), probe 56; IL-1 β : 5'-TTGACGGACCCCAAAAGAT-3' (forward), 5'-GAAGCTGGATGCTCTCATCTG-3' (reverse), probe 26; IFN- γ : 5'-ATCTGGAGGAAGTGGCAAAA-3' (forward), 5'-TTCAAGACTTCAAAGAGTCTGAGGTA-3' (reverse), probe 21; IL-6: 5'-GCTACCAAACTGGATATA-ATCAGGA-3' (forward), 5'-CCAGGTAGCTATGGTACT-CCAGAA-3' (reverse), probe 6; and IL-10: 5'-GCTCCTAGAGCTGCGGACT-3' (forward), 5'-TGTTGTCCAGCTGGT-CCTTT-3' (reverse), probe 41.

qRT-PCR for cytokines from ear sheets

Ear sheets were prepared as described (6) 1, 4.5 and 24 h after ear painting and stored in liquid nitrogen. Frozen ear skin was crushed in liquid nitrogen and mixed with a solution of 1600 μ l solution D (47 g guanidinium thiocyanate, 0.5 g *N*-lauroyl sarcosine, 2.5 ml sodium citrate [1M, pH 7], 700 ml β -mercaptoethanol, ad 100 ml H₂O) and 300 μ l 2 M sodium acetate. Two millilitres of water-saturated phenol and 400 μ l of a chloroform–isoamyl alcohol solution (49:1) were added to the suspension followed by mixing and incubation on ice for 10 min. The mixture was centrifuged (3000 g, 15 min, 4°C) while the upper aqueous phase was removed thereafter. Two volumes of isopropanol were added to this supernatant and incubated over night at –20°C. The suspension was centrifuged (3000 g, 30 min, 4°C) followed by washing the pellet with ethanol (80%) and an additional centrifugation (3000 g, 15 min, 4°C). The pellet was solubilized in 300 μ l lysis buffer (Stratagene). The RNA was further purified with the Absolutely RNA Miniprep Kit from Stratagene.

RNA concentration was measured by UV extinction (260 and 280 nm) while RNase free water served as a reference. cDNA was prepared with the Expand Reverse Transcriptase Kit (Roche) using oligo-dT-primers. qRT-PCR was performed with TaqMan Master and Lightcycler (Roche).

Cytokine measurement by ELISA

Ear sheets were prepared as described (6) 24 h after ear painting with contact allergens and stored on ice. Briefly, the epidermal side was divided from the cartilage side, and both parts were incubated in RPMI medium for 24 and 48 h, respectively. The supernatant was removed and analysed by ELISA.

ELISA for IFN- γ was performed as described (33). Mouse IL-10 ELISA was carried out according to manufacturers' instructions using OptEIA ELISA Sets from BD Biosciences. Plates were measured in an MWGt Sirius HT-TRF instrument (MWG, Ebersberg, Germany), and concentra-

tions were calculated using BIO-TEK KC⁴ v3.1 software (Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany).

Induction of contact hypersensitivity

Cytokine expression was analysed in C57BL/6 mice. CHS was induced as described (24). In the first group, mice were sensitized by epicutaneous application of 100 μ l 7% TNCB in acetone or acetone as vehicle control on the shaved abdominal skin on day 0. Some mice were pretreated with Central European or Spanish Arnica tincture (undiluted) or received EtOH as solvent control on days –2, –1 and 0 on the shaved abdominal skin as described (27). Mice were challenged on day 5 with 20 μ l of 1% TNCB or 20 μ l of the Arnica tinctures applied on both ears. In the second (non-sensitized) group, mice were only painted on both ears with 20 μ l of 1% TNCB or 20 μ l of the Arnica tinctures.

Adoptive P14 T-cell transfer and Skin-Homing Assay

The assay was performed as described (6). A total of 1.5×10^6 spleen cells of P14 mice in 200 μ l PBS were injected i.v. into C57BL/6 recipient mice on day 0. Bone marrow-derived DC (BMDC) were prepared as described (5). DC were incubated for 1 h in RP-10 with 1 μ M peptide GP33 (34) (BioChip Technologies GmbH, Freiburg, Germany) at 37°C. After 3 washes with PBS, 3×10^5 DC in 100 μ l PBS were used for i.c. injection into two sites of the shaved abdominal skin on day 0 and day 2 after P14 T-cell transfer. On day 9 after adoptive P14 transfer, skin inflammation was induced by ear painting with 20 μ l of 1% TNCB in acetone or Arnica tinctures in EtOH. Ear sheets were prepared 24 h later and incubated overnight in RP-10. Emigrated cells were counted, stained with antibodies for the expression of CD8 α and the transgenic P14 TCR V α 2 and were analysed by flow cytometry.

The emigrated cells (5×10^5 – 1×10^6) were stained for 20 min with 0.5–1 μ g biotinylated mAb in 100 μ l PBS/2% FCS/0.02% NaN₃ (FACS buffer) on ice. Cells were washed three times in FACS buffer, and FITC-, PE- or biotin-conjugated secondary antibodies as well as streptavidin-Cy-Chrome® (BD PharMingen) were added for 20 min on ice. Samples were washed 3 $\times$ and resuspended in 200 μ l 1% paraformaldehyde. Data were acquired on a FACScan instrument and analysed using CellQuest Software (BD Biosciences).

Statistical analysis

Statistical analysis was performed using the Origin 7.0 (North Hampton, MA, USA) and Microsoft Excel software (Unterschleißheim, Germany). Data are reported as

means $\pm$ SD and analysed using an independent Student's *t* test (two groups). A *P*-value < 0.05 is considered statistically significant.

Results

Induction of innate and effector cytokines by TNCB and Arnica tinctures

To investigate the inflammatory potential of Spanish and Central European Arnica tinctures in comparison to the strong contact allergen TNCB, the induction of pro- and anti-inflammatory cytokines was determined by qRT-PCR (Fig. 1a, b). Undiluted Central European (CE) or Spanish (SP) Arnica tinctures or TNCB were used for sensitization and challenge of C57BL/6 mice for sensitization. TNCB induced a strong increase in the pro-inflammatory cytokines IL-1 β and IL-6 in mice following sensitization while no induction of the effector cytokine IFN- γ was observed. The strongest effect was observed after 4.5 h (Fig. 1a). After sensitization and challenge, a similar increase in the expression of the pro-inflammatory cytokines by TNCB, which was twofold or even threefold higher compared to non-sensitized mice, was demonstrated (Fig. 1b). IFN- γ was significantly increased, which indicates the induction of a Tc1 response to TNCB as described (33). The Arnica tinctures did not induce a significant increase in IL-1 β , IL-6 or IFN- γ at all, neither after sensitization (Fig. 1a) nor after sensitization and challenge (Fig. 1b). Interestingly, the expression of the anti-inflammatory cytokine IL-10 was enhanced by TNCB and the Spanish tincture in sensitized and challenged mice, but the increase was not significant. Taken together, tinctures from *Arnica montana* do not increase the expression of pro-inflammatory cytokines.

Induction of the anti-inflammatory cytokine IL-10 by TNCB and Arnica tinctures

To further investigate the influence of the Arnica tinctures on IL-10 protein expression in sensitized versus sensitized and challenged mice, supernatants from ear sheets of tincture-treated mice were analysed by ELISA (Fig. 2). Mice sensitized with the Spanish tincture showed no IL-10 increase after 24 h but a weak increase of IL-10 (about 1.5-fold, 100–430 pg/ml) compared to TNCB (about threefold, 290–440 pg/ml) after 48 h (Fig. 2a). Sensitization and challenge with the Spanish tincture resulted in a significant IL-10 increase compared to solvent control (EtOH) or Central European tincture, which was similar to that of TNCB after 24 h (about threefold, 140–240 pg/ml) (Fig. 2b). The Central European tincture did not reveal IL-10 induction above the ethanol control. These data confirm the results from qRT-PCR (Fig. 1) and show that the Spanish but not the Central European tincture increases the expression of IL-10 also on the protein level.

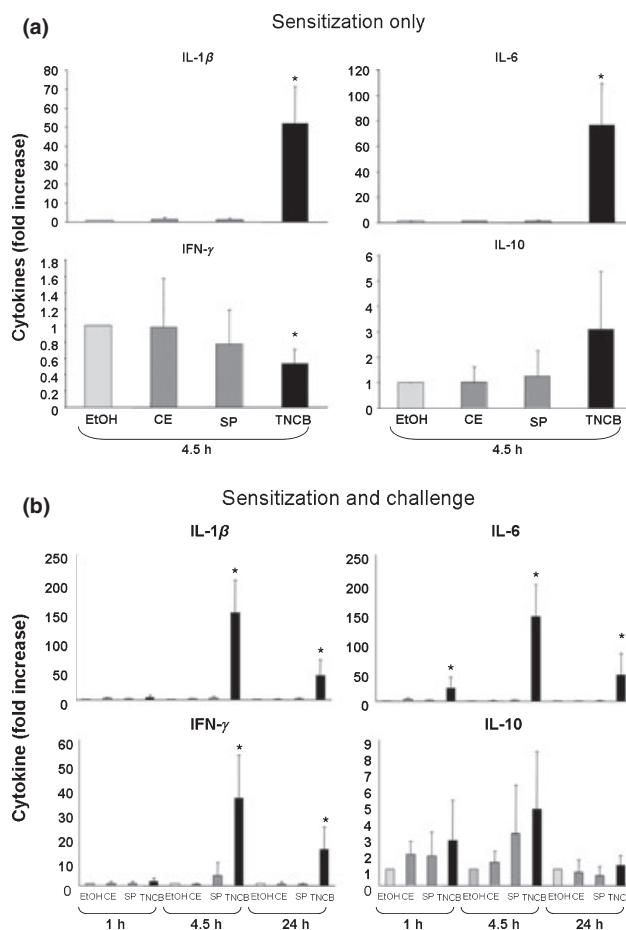


Figure 1. Cytokine mRNA expression induced by TNCB and Arnica tinctures before and after challenge. (a) Sensitization only: Ears of C57BL/6 mice were painted with ethanol (EtOH), undiluted Central European (CE) or Spanish (SP) Arnica tinctures or 1% TNCB/acetone. After 4.5 h, mRNA was extracted from ear sheets, and the induction of cytokines was analysed by qRT-PCR. Ethanol treatment was used as negative control. The results represent the fold-increase in cytokine mRNA with the ethanol value set as 1. Data correspond to the average of 3 independent experiments with two mice per group $\pm$ SD. (b) Sensitization: C57BL/6 mice were treated with EtOH or undiluted CE or SP tinctures on day -2, -1 and 0 on the abdomen. Abdominal treatment with 7% TNCB/acetone on day 0 served as positive control. Challenge: ears were painted with ethanol, undiluted CE or SP tinctures or 1% TNCB/acetone on day 5. After 1, 4.5 and 24 h, ear sheets were prepared, and cytokine mRNA analysis was performed as described in (a). * *P*-value < 0.05 .

Potency of TNCB and Arnica tinctures to induce recruitment of skin-homing effector T cells

To analyse whether the magnitude of the pro-inflammatory responses induced by the Arnica tinctures or by TNCB is reflected by the efficiency of effector T-cell recruitment to the skin, we used an *in vivo* model. The skin-homing assay (6) allows to quantify the inflammation-dependent immigration of skin-homing CD8 $^{+}$ P14 effector T cells into

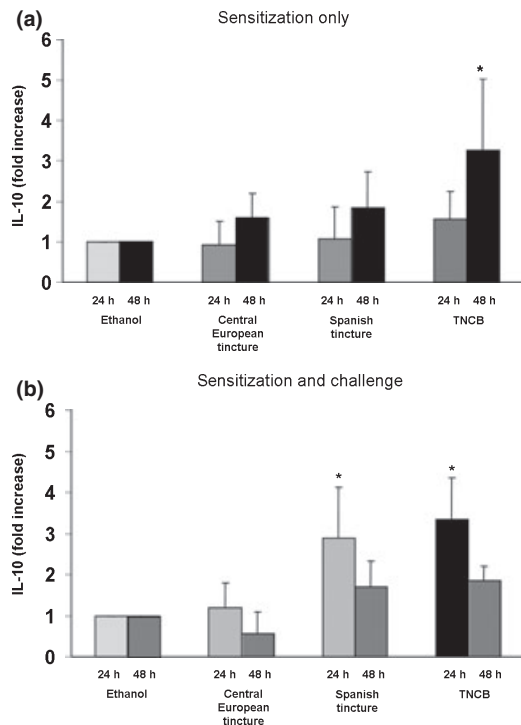


Figure 2. IL-10 protein expression induced by TNCB and Arnica tinctures. (a) Ears of C57BL/6 mice were painted with ethanol, undiluted Arnica tinctures or 1% TNCB/acetone or (b) treated abdominally with ethanol, the undiluted tinctures or 7% TNCB/acetone on days -2, -1 and 0. Challenge was carried out by treatment of the ears with the undiluted tinctures or 1% TNCB/acetone on day 5. After 24 h, ear sheets were prepared and incubated in culture medium for 24 or 48 h, respectively. IL-10 concentrations were measured by ELISA. The results represent the fold-increase with the ethanol value set as 1. Data present the average of three independent experiments with 1 mouse per group. Values with significance level of $P < 0.05$ to the negative control are marked (*).

contact allergen-treated skin. Following T-cell priming with GP33-pulsed BMDC, emigration of the infiltrated T cells was analysed 24 h after ear painting with TNCB or Arnica tinctures, which triggers the release of cytokines and chemokines that recruit effector T cells expressing skin-homing receptors from the blood into the skin (6). The Arnica tinctures induced P14 T-cell infiltration, which was about fivefold lower compared to that induced by TNCB (Fig. 3). These data reveal that the Arnica tinctures have a low potential for the induction of skin inflammation as reflected by the recruitment of effector T cells compared to the strong contact sensitizer TNCB. This is in line with our findings regarding their low potential to induce CHS (27). The isolated SLs from Arnica, helenalinisobutyrate and 11,13 α -dihydrohelenalinmethacrylate were also studied but failed to show any significant effects above solvent control (data not shown). This is most likely due to the inefficient skin penetration of the SLs compared to the Arnica tinctures (35) also reflected in CHS (27).

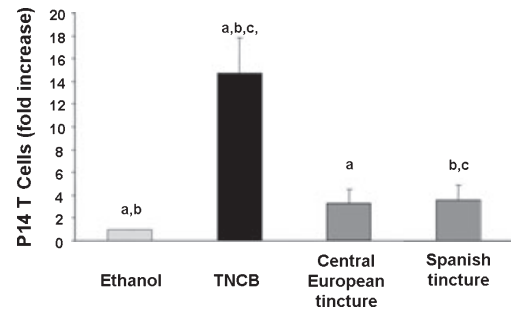


Figure 3. Skin inflammation induced by TNCB and Arnica tinctures. TCR transgenic CD8⁺ P14 T cells were transferred i.v. into C57BL/6 mice on day 0 and activated *in vivo* by i.c. injection of GP33-pulsed DC on days 0 and 2. On day 9, 1% TNCB, Central European tincture (undiluted) and Spanish tincture (undiluted), respectively, were applied on ear skin. Ear sheets were prepared 24 h later and incubated in media for 24 h. The emigrated cells were counted, and P14 T cells were quantified by flow cytometry. The results represent the fold-increase in emigrated P14 T cells with the ethanol value set as 1. The value for TNCB corresponds to 30 000–115 000 emigrated P14 T cells per ear depending on the experiment. Data present the average of three independent experiments with three or two mice per group. Values with significance level of $P < 0.05$ are marked with the same letter.

Dose-dependent cytokine induction and T-cell recruitment by TNCB

To verify the specificity of the assays, the concentration dependence of the IL-1 β induction and of the P14 effector T-cell homing was analysed (Fig. 4). Both IL-1 β induction and recruitment of skin-homing P14 T cells increased with increasing doses of TNCB. This indicates a specific, dose-dependent innate immune response to TNCB.

Discussion

Skin inflammation is essential for the sensitization to contact allergens and also plays an important role in the recruitment of effector cells in the elicitation phase. Contact allergens induce skin inflammation by the activation of the innate immune system. This is achieved by triggering of innate immune and stress responses employing pathways that are also used by pathogens (4). Recent evidence was provided that Toll-like receptors (TLR) and NOD-like receptors play a role in CHS, as well as oxidative stress responses (4,22). Thus, contact allergens can induce ligands for TLR2 and TLR4 (15) as well as activate the NLRP3 inflammasome (36,37) and the production of reactive oxygen species (38). TLR triggering results in the production of pro-inflammatory cytokines including IL-6 and IL-12, as well as production of pro-IL-1 β and pro-IL-18. These are important mediators in CHS. Pro-IL-1 β and pro-IL-18 are processed to their mature and secreted forms by the inflammasome, a cytosolic protein complex containing the NLR NLRP3 and the adaptor protein ASC (39). The

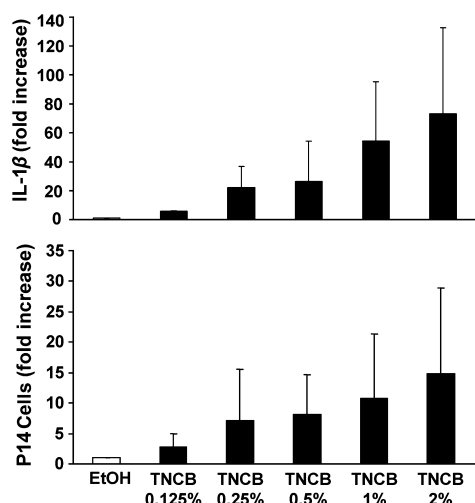


Figure 4. Dose-dependent increase in IL-1 β or T cell homing to inflamed skin. C57BL/6 mice were treated with different concentrations of TNCB. The qRT-PCR analysis of IL-1 β induction (upper panel) and the skin-homing assay (lower panel) was performed as described in Figs. 1 and 3, respectively. Data present the average of three independent experiments with three or two mice per group $\pm$ SD.

inflammasome is activated by contact allergens and then activates caspase-1, which processes pro-IL-1 β and pro-IL-18. The absence of cytokines like IL-1 β , IL-18, IL-6 or IFN- γ impairs or abrogates the induction of CHS in mice (40–44). On the other hand, the influence of anti-inflammatory cytokines like IL-10 produced by regulatory T cells, B cells and mast cells also causes the inhibition of CHS development (23–25,45). Analysis of the cytokine spectrum in the different phases of CHS may allow the identification of a contact allergen-specific cytokine signature or signatures that will be useful in the identification of potential contact allergens. Moreover, Dearman et al. could show that expression of cytokines like IFN- γ , IL-4 and IL-12 can be considered to differentiate between contact and respiratory allergens (13). It is conceivable to expect that the extent of the inflammatory response induced by contact allergens in the skin correlates with the extent of cytokine production and the ACD response.

We have therefore used the CHS model to study the qualitative and quantitative aspects of the induction of pro-inflammatory cytokines as one of the first and essential steps in the development of CHS using weak contact allergens, the SLs from Arnica (27), and the strong contact allergen TNCB. We observed that weak contact sensitizers like Arnica tinctures only cause a weak enhancement of pro-inflammatory cytokines like IL-1 β , IL-6 or IFN- γ , whereas the contact sensitizer TNCB strongly enhances the expression of these cytokines. This is in line with the capacity of SLs to suppress NF- κ B activation as shown by us for keratinocytes and DC (27). Interestingly, the Spanish

Arnica tincture causes an enhanced expression of the anti-inflammatory cytokine IL-10, which was similar to the level caused by TNCB. While the enhanced expression of pro-inflammatory cytokines may overcome the IL-10 effect in the case of TNCB, increased IL-10 expression in the absence of high levels of pro-inflammatory cytokines may explain the weak allergenic potential in the case of the Spanish tincture. Interestingly, the Spanish tincture holds a weaker potential to induce CHS than the Central European tincture that lacks this enhancement of IL-10 (27). It is further important that IL-6 can suppress the activity of CD4+ CD25+ regulatory T cells (46) and its absence impairs CHS responses (42). Thus, a lack of IL-6 causes a regulatory barrier for allergic responses. We observed that, similar to IL-1 β , Central European tincture and Spanish tincture failed to induce significant IL-6 production. These data underline our previous findings regarding their failure to cause CHS and support the notion that SLs are rather weak contact allergens. We have shown that they exert dominant anti-inflammatory effects *in vivo* in the CHS model that even allow to suppress CHS to TNCB (27).

The results observed on the mRNA and protein level are concordant to the observations on the *in vivo* studies in the skin-homing assay. Skin-homing effector T cells were only recruited efficiently when skin inflammation was induced with TNCB. Here, the tinctures revealed a weak pro-inflammatory effect that could not be observed in the qRT-PCR. The T-cell homing response was dependent on the dose of TNCB and correlated well with the extent of the pro-inflammatory response as detected by qRT-PCR analysis of IL-1 β induction. This shows that it is important to take into account a broader spectrum of cytokines and also chemokines such as CCL17 and CCL27 that play a role *in vivo*, e.g., for the recruitment of effector and memory T cells to the skin. However, there was a good correlation of the *in vitro* results with the allergenic and pro-inflammatory *in vivo* potency (27).

We have demonstrated that the potency of the weakly contact allergenic SLs from Arnica in comparison to the strong contact allergen TNCB is reflected by their ability to induce pro-inflammatory cytokines. In the case of TNCB, a dose-dependent efficiency of the recruitment of skin-homing effector T cells to the inflamed skin was observed. These findings imply that qualitative and quantitative cytokine profiling may be a useful tool to identify putative contact allergens before their use in consumer products (5,13). It can also be used to roughly differentiate weak and strong contact allergens.

EU legislation now prohibits the use of the local lymph node assay (47,48) for the assessment of the sensitizing potential of chemicals. Therefore, the development of *in vitro* assays that can identify contact allergens and allow potency assessment is an important issue for hazard

identification and risk assessment to reduce and eventually replace animal testing.

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