

Extended DNFB-induced contact hypersensitivity models display characteristics of chronic inflammatory dermatoses

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Abstract: Despite recent developments, there is a high medical need for new treatment options for chronic inflammatory dermatoses like allergic contact dermatitis (ACD) and psoriasis. Particularly, more predictive skin inflammation models are required to facilitate the process of drug discovery. Murine contact hypersensitivity (CHS) models adequately reflect ACD and are also used to characterize therapeutic approaches for psoriasis. Using the hapten 2,4-dinitrofluorobenzene (DNFB), we established new subacute and subchronic DNFB-induced CHS models in C57BL/6 mice, which more closely reflect the characteristics of chronic T-cell-dependent inflammatory dermatoses as pronounced keratinocyte proliferation, strong hypervascularization, immune cell infiltration and overexpression of T cell and inflammatory cytokines. For the subacute DNFB model, we demonstrated anti-inflammatory activity of the

glucocorticoid, prednisolone, as well as of neutralization of TNF α , IL-12/IL-23 or IL-18. In the subchronic DNFB-induced CHS model, deficiency for MyD88 and IL-12/IL-35 p35 chain but not IL-12/IL-23 p40 chain led to decreased skin inflammation. Furthermore, as exemplified by the dose-dependently effective therapeutic prednisolone treatment, the subchronic model allows the continuous therapy of a pre-established stable contact dermatitis. Altogether, prolonged DNFB-induced mouse CHS models closely reflect ACD sensitive to glucocorticoids as standard therapy, reveal a more chronic skin inflammation and are responsive to cytokine antagonization.

Key words: 2,4-dinitrofluorobenzene – allergic contact dermatitis – mouse model

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Introduction

Allergic contact dermatitis (ACD) is a highly prevalent, potentially chronic T-cell-mediated disease caused by repeated skin exposure to contact allergens. Pathophysiological understanding of ACD is derived from animal models referred to as contact hypersensitivity (CHS), in which the skin inflammation is induced by painting a hapten onto the skin. The hapten triggers danger signals and inflammatory cytokines and is taken up by activated dendritic cells, which migrate to the skin-draining lymph nodes and prime naïve T cells during the sensitization phase. Upon re-exposure to the hapten in the elicitation phase, the allergen-specific T cells are recruited into the skin. The disease becomes manifest because of the cytotoxicity of CD8⁺ effector T cells and the interaction of recruited inflammatory cells with keratinocytes and stromal cells. Finally, the inflammatory response is resolved in the resolution phase (1–4).

Overall, murine CHS reactions seem to adequately reflect human ACD. The most commonly used acute CHS models, however, have limitations with regard to the treatment in a therapeutic setting and to the reflection of the immunopathophysiology of chronic ACD (5). Accordingly, novel more extended CHS models are necessary.

Besides its usage as model for human ACD, CHS is also used in the pharmaceutical research for psoriasis. Psoriasis is a T-cell-mediated chronic relapsing inflammatory skin disease that is

characterized by exaggerated keratinocyte proliferation and strong hypervascularization, immune cell infiltration and cytokine increases reflecting the interplay between keratinocytes and immune cells (6,7). Comparably profound alterations can be found in chronic ACD (8,9).

For psoriasis, a variety of rodent models exist which, however, have distinct limitations (5,10,11). So, mice homozygous for *asebia* or flaky skin mutations exhibit a psoriasis-like phenotype, which, however, develops independently from T cells. Relevant transgenic mice are based on single-gene manipulation, whereas psoriasis likely results from interactions of multiple genetic susceptibility loci (7). Humanized psoriasis models using xenotransplantation of diseased skin are restricted by their complexity and the restrained skin disposability (12). Because of the limitations of assigned psoriasis models and because of the existing common mechanistic aspects of psoriasis and ACD (6), in pharmaceutical research for psoriasis, the usage of CHS models is quite common (13).

Rodent CHS models are easy to handle, highly reproducible, inexpensive and well characterized. Dermal sensitizers like 2,4,6-trinitrochlorobenzene (TNCB), oxazolone or 2,4-dinitrofluorobenzene (DNFB) are commonly used to stimulate an acute CHS, which exhibits a T-cell-dependent inflammatory response to cutaneous sensitization and subsequent challenges. A variety of CHS models have been established using different sensitiz-

ers, sensitization/challenge schedules and mouse strains (14,15). The dermal sensitizer DNFB is commonly used to evoke a type 1 cytokine-dominated skin inflammation in C57BL/6 mice (16).

We have developed subacute and subchronic DNFB-induced mouse CHS models to better reflect the subacute to chronic pathology of ACD and psoriasis. Our models display cutaneous characteristics of prolonged T-cell-dependent inflammatory dermatoses as chronic T cell infiltration and cytokine increase, hyper-vascularization, keratinocyte proliferation and acanthosis. Moreover, the subchronic DNFB model allows the therapeutic treatment of a pre-established stable and non-necrotic skin inflammation. Importantly, our newly developed subacute and subchronic DNFB models reflect the sensitivity of human ACD to glucocorticoid therapy (8) as well as to some extent the sensitivity of psoriasis to antagonization of inflammatory cytokines like TNF α and IL-12/IL23 (17).

Methods

Animals

For all experiments, mice with C57BL/6 background and a body weight of 20–26 g were used. Wild-type and nude mice (nu/nu) were obtained from Charles River (Sulzfeld, Germany), mice deficient for IL-12/IL-35 p35 chain or IL-12/IL-23 p40 chain from Roche (Palo Alto, CA, USA) and MyD88-deficient mice from Max Planck Institute for Infection Biology (Berlin, Germany). Mice were housed according to institutional guidelines with access to food (pelleted diet) and water *ad libitum*. Animal experiments have been approved by the governmental administration of Berlin (Berlin, Germany).

Schedules for mice sensitization and challenges

In the acute, subacute and subchronic DNFB-induced CHS models, mice were sensitized with 25 μ l of 0.5% DNFB on the shaved flank skin on day 0 and day 1. DNFB (Sigma, Seelze, Germany) was dissolved in acetone and olive oil (4:1, v/v) (Sigma) immediately prior to application. Challenges with 10 μ l of 0.3% DNFB on the dorsum of both ears were performed on day 5 (acute model) and on day 5, 6 and 7 (subacute model). In the subchronic model, animals received 10 μ l of 0.3% DNFB on day 5, 6 and 7 and subsequently 10 μ l of 0.1% DNFB on the ears on days 8–13. End-point parameters were taken at 24 h after the last DNFB challenge, that is on days 6, 8 and 14 in acute, subacute and subchronic DNFB models, respectively. In each model, a solvent control group was exposed to acetone and olive oil (4:1, v/v) throughout the duration of the experiment. Ear thickness over the time course was determined with a custom-built micrometer (Bayer Pharma AG, Berlin, Germany).

Treatment and drugs

Prednisolone (Sigma) was dissolved in dimethylsulfoxide (Merck, Darmstadt, Germany), ethanol (96%; Sigma) and peanut oil (Sigma) (5:3:92, v/v/v). In the subacute DNFB model, mice were subcutaneously treated with 30 mg/kg prednisolone 2 h before every challenge. In the subchronic model, 3 mg/kg and 30 mg/kg prednisolone were given orally 2 h before challenges on days 10–13.

Cytokine neutralization studies were performed using 300 μ g rabbit anti-mouse TNF α IgG1 antibody (Thermo Fischer, Rockford, IL, USA), 100 μ g rat anti-mouse IL-12p40 IgG2a antibody (clone C17.8; NatuTec, Frankfurt am Main, Germany), 100 μ g rat

anti-mouse IL-18 antibody (93-10C; Invitrogen, Carlsbad, CA, USA), rabbit IgG (Serotec, Duesseldorf, Germany), rat IgG2a (NatuTec) and rat IgG1 (BD Bioscience, Heidelberg, Germany) for the respective controls. Antibodies and isotype controls were intraperitoneally administered 2 h before first challenge on day 5.

Determination of immune mediators

For determination of myeloperoxidase activity as a parameter for cutaneous granulocyte infiltration, the ears were mechanically homogenized in 2 ml homogenization buffer (hexadecyltrimethyl ammonium bromide/morpholinopropan sulphonic acid; Sigma) and centrifuged at 25 000 g and 12°C for 20 min. The myeloperoxidase activity assay in supernatants was performed as we recently described (18).

For assessment of cutaneous cytokine levels, ears were mechanically homogenized in 2 ml RPMI1640 medium (Biochrom AG, Berlin, Germany) at 4°C and centrifuged at 25 000 g and 4°C for 20 min. Cytokine concentrations in supernatants were determined using the Proinflammatory 7-plex culture kit and the TH1/TH2 9-plex tissue culture kit and MesoScale SI6000 equipment (all from MesoScale Discovery, Gaithersburg, MD, USA).

RNA isolation from skin and quantitative real-time RT-PCR analyses

RNA from skin specimens (ears) was extracted and purified using Invisorb RNA Kit II (Invitex, Berlin, Germany). Immediately after sample homogenization, a proteinase K digestion (4 mg/ml) (BD Bioscience) was performed. Purified RNA was qualified and quantified with the Agilent Bioanalyzer 2100 system with RNA 6000 Nano Assay Kit (Agilent Technologies, Boeblingen, Germany). cDNA was synthesized using reverse transcription reagents and GeneAmp[®] PCR System 9700 (Applied Biosystems, Darmstadt, Germany). RT-PCR of cDNA was performed on a 7900HT Sequence Detection System (Applied Biosystems) under the following thermal conditions: 10 min 95°C, 40 cycles, 15 s 95°C and 1 min 60°C. The following murine genes were analysed using Assays on demand from Applied Biosystems: HPRT, IFN γ , TNF α , IL-1 β , IL-2, IL-4, IL-10, IL-12p35, IL-12p40, IL-13, IL-17, IL-18, IL-20, IL-22, IL-23p19, IL-24, IL-27 α , EBI3 and T-bet. The reactions were performed using a universal PCR Master Mix without UNG (Eurogentec, Koeln, Germany). Expression of target genes was relatively quantified as fold expression of the house-keeping gene, HPRT.

Immunohistochemistry

For immunohistochemistry, half an ear was embedded in freezing medium (Leica, Wetzlar, Germany), cut in 6- μ m-thick sections (Cryostat CM3050S; Leica Microsystems, Wetzlar, Germany) and fixed with acetone. Non-specific activity for peroxidase staining was blocked with H₂O₂ (Merck). Non-specific binding sites were blocked with goat serum (Dako, Hamburg, Germany). Monoclonal rat antibodies against mouse CD31 (clone ER-MP12; Acris, Herford, Germany), Ki67 (TEC-3; Dako), MHCII (I-Ad) (M5/114.15.2), CD4 (H129.19) or CD8 (53-6.7) (all from BD Bioscience) were used for characterization of vessel formation, proliferation and infiltrating immune cells by indirect staining with a biotinylated goat anti-rat secondary antibody (BD Bioscience). Labelled cells were visualized with horse radish peroxidase conjugated with streptavidin (Dako). Sections were counterstained with haematoxylin (Sigma). Pictures were taken by using MIRAX

MIDI Slide Scanner and Viewer software (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Unless otherwise noted, data are given as mean $\pm$ standard error of the mean (SEM). Statistical significance was estimated with

Mann–Whitney *U*-test after proving significance in Kruskal–Wallis test (SigmaStat 3.0, IBM SPSS, Muenchen, Germany). An error probability (*P*) of <0.05 was considered as significant. Percentage values given are the relative inhibition of inflammation in the treatment group as compared to the increase of inflammation from solvent to the DNFB group (100%).

Results

Comparison of skin inflammation in acute, subacute and subchronic DNFB-induced mouse CHS models

A variety of skin inflammation parameters (Fig. 1 and Figure S2) and immunohistochemistry (Figure S1) were compared for the endpoints of acute (one challenge on day 5 after sensitization), subacute (three challenges) and subchronic (nine challenges) DNFB-induced CHS models in C57BL/6 mice.

Almost all ear inflammation parameters increased with model prolongation. So, the subchronic DNFB model exhibited the strongest enhancement of ear weights, ear thickness and cutaneous granulocyte infiltration as assessed by peroxidase activity (Fig. 1a and Figure S2a). Importantly, however, all models, including the subchronic DNFB model, which after an induction phase displays strong and stable skin inflammation for about 6 days, showed a non-necrotic skin inflammation.

In immunohistochemistry (Figure S1), the subchronic DNFB-induced CHS model exhibited a pronounced cutaneous immune cell infiltration and morphological characteristics of chronic eczema. Dermal infiltration by MHCII-positive cells was found in all three models but was more pronounced in the subacute and subchronic DNFB-induced CHS models than in the acute model. Moreover, dermal infiltration by CD4⁺ T helper cells and the epidermal infiltration by CD8⁺ T cells were rather weak in the acute and strongest in the subchronic DNFB model. Finally, only the subchronic DNFB-induced CHS model clearly showed morphologic characteristics of chronic inflammatory dermatoses such as acanthosis, pronounced keratinocyte proliferation and strong hypervascularization as demonstrated by Ki67 and CD31 staining, respectively.

When analysing the cytokine protein levels in homogenates from inflamed skin, the highest values were observed in the subchronic model (Fig. 1b and Figure S2b). This applies to proinflammatory cytokines (TNF α , IL-1 β , IL-12p40), to the counter-regulatory anti-inflammatory cytokine IL-10 and to lymphokines, including IL-2, IL-4, IL-5 and IFN γ . No significant increase, however, was found for IL-17 in inflamed skin although its increase in the elicitation phase of human ACD has been described (19). However, the IL-17 values tended to be higher in the acute and subacute DNFB models (data not shown).

The subchronic DNFB model has been further characterized for cutaneous cytokine mRNA expression. Significant increases in comparison with the solvent group were observed for IFN γ , TNF α , IL-1 β , IL-4, IL-10, IL-12p35, IL-13, IL-24, IL-27 and EBI3 (Figure S3).

The increased expression of the type 1 cytokines in the subchronic DNFB model corresponded well to a T-bet expression that incrementally increased with model prolongation (Figure S3 and data not shown). However, as the Th2 cytokines, IL-4, IL-5, and IL-13 also exhibited considerable increases on protein and/or mRNA level, a mixed Th1/Tc1 and Th2 cytokine profile results for the subchronic DNFB-induced CHS model.

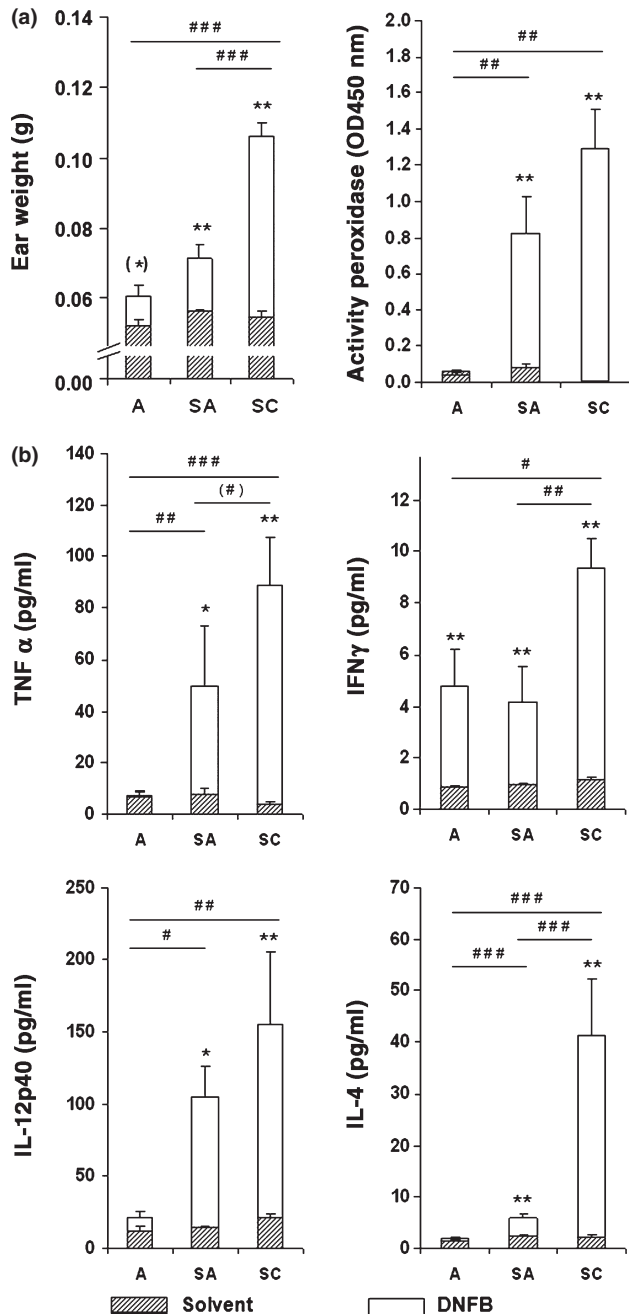


Figure 1. Comparison of inflammation parameters in DNFB-induced mouse contact hypersensitivity models. (a) Ear weight, myeloperoxidase activity in ear homogenates, and (b) cytokine levels in ear homogenates were determined at the endpoints in acute (A) (day 6, $n = 6$), subacute (SA) (day 8, $n = 7$) and subchronic (SC) (day 14, $n = 10$) DNFB models in female C57BL/6 mice. (* $P < 0.1$, ** $P < 0.05$, *** $P < 0.01$ versus solvent group; (#) $P < 0.1$, # $P < 0.05$, ### $P < 0.01$, #### $P < 0.001$ comparison of DNFB groups between the three models.

Kinetic characterization and anti-inflammatory treatment in the subacute DNFB-induced mouse CHS model

In the subacute model, three daily DNFB challenges were used to trigger skin inflammation in sensitized mice. Kinetics of inflammation was followed on days 5 (prechallenge), 6, 7 and 8 (Figure S4a). Whereas ear weights showed a clear increase on day 6, ear thickness and cutaneous granulocyte infiltration as assessed by myeloperoxidase activity strongly increased on days 7 and 8. The gene expression of inflammatory cytokines, $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-12/IL-35 p35-chain, progressively increased in inflamed skin. In contrast, $\text{IFN}\gamma$ showed its maximum on day 6, and IL-23p19 expression was significantly increased exclusively on day 8. In addition, the T cell dependence of the subacute DNFB-induced CHS model was investigated in T-cell-deficient athymic homozygous nu/nu mice, which failed to develop an inflammatory response to DNFB (Figure S4b).

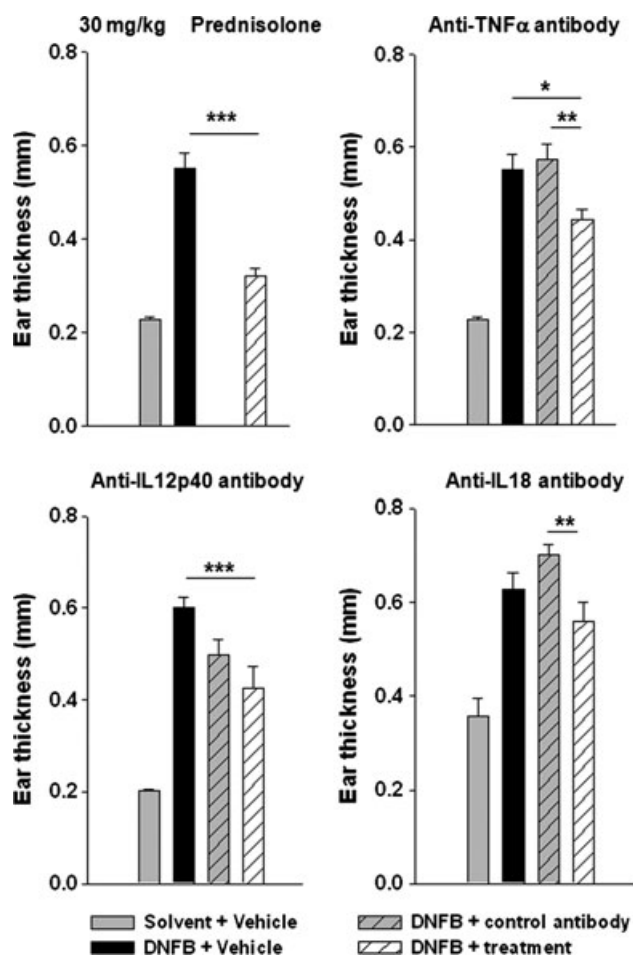


Figure 2. Anti-inflammatory treatments in subacute DNFB-induced mouse contact hypersensitivity model. Ear thickness on day 8 was determined in subacute DNFB model in female C57BL/6 mice ($n = 10$ per group) undergoing daily prednisolone treatment (30 mg/kg) before each DNFB challenge or $\text{TNF}\alpha$, IL-12/IL-23 p40-chain and IL-18 antagonization by application of neutralizing antibodies on day 5 before the first DNFB challenge. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

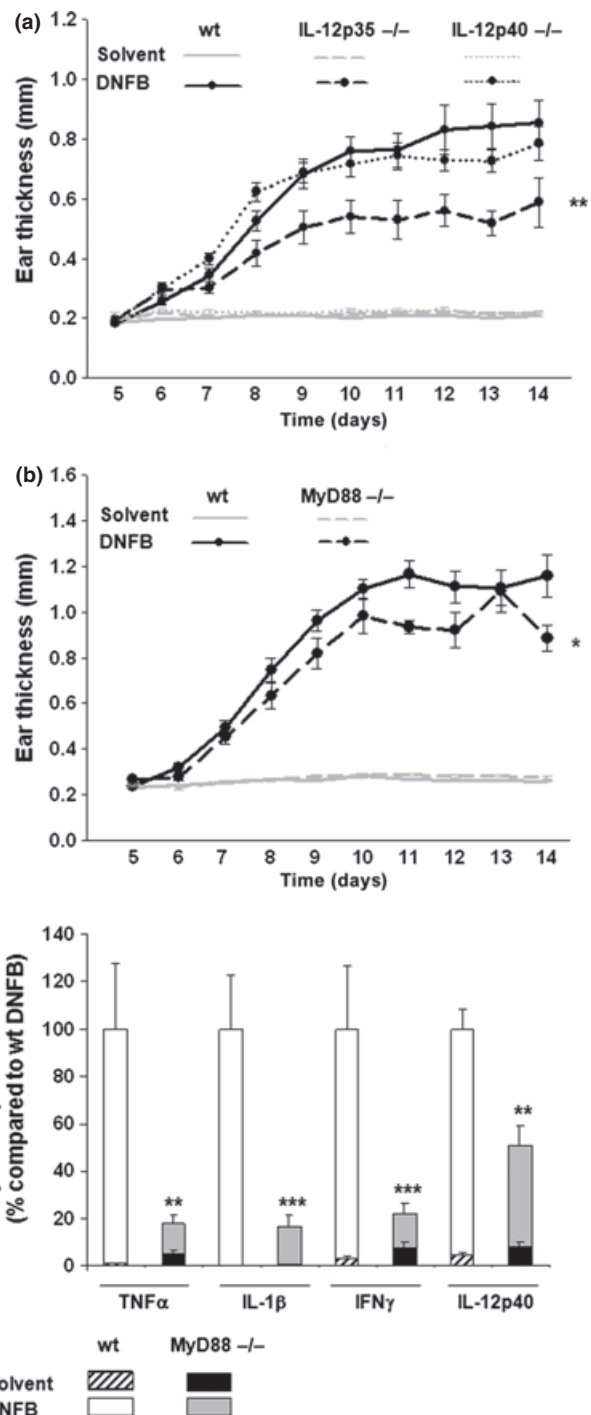


Figure 3. Subchronic DNFB-induced contact hypersensitivity model in gene-deficient mice. (a) Deficiency for IL-12/IL-35 p35 chain but not for IL-12/IL-23 p40 chain diminished skin inflammation as assessed by increase in ear thickness in female C57BL/6 mice (wild-type $n = 10$, p35-deficient $n = 12$, p40-deficient $n = 9$). (b) Ear inflammation was diminished in female MyD88-deficient in comparison with wild-type C57BL/6 mice (solvent group wildtype mice $n = 6$; other groups $n = 8$). Cytokine levels in ear homogenates are shown as percentage of the mean values in the DNFB wild-type group ($\text{TNF}\alpha$ 28.50 ± 7.8 pg/ml, $\text{IL-1}\beta$ 953.6 ± 220.1 pg/ml, $\text{IFN}\gamma$ 5.67 ± 1.5 pg/ml, IL-12p40 4.2 ± 0.4 pg/ml). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Next, we tested the effect of anti-inflammatory therapies on skin inflammation in the subacute DNFB-induced CHS model. The endpoint data for ear thickness are given in Fig. 2.

Glucocorticoid treatment by application of 30 mg/kg prednisolone at 2 h before the daily DNFB challenges significantly inhibited ear swelling on day 7 (80%, not shown) and day 8 (71%). Furthermore, neutralization of TNF α , IL-12/IL-23 p40 and IL-18 by antibody applications on day 5 led to a moderate inhibition of ear swelling on day 8, which was significant in comparison with the DNFB and/or IgG control groups. In contrast to prednisolone treatment, however, almost no inhibitory effect of cytokine neutralization was observed before day 8 (data not shown). This indicates that prolonged CHS models might better than acute models reflect the situation in chronic inflammatory skin diseases with regard to the antagonization of inflammatory cytokines.

Characterization of the subchronic DNFB-induced CHS model in gene-deficient mice

As determined by daily ear thickness measurements, the subchronic DNFB-induced CHS model, after a 4-day induction period, showed a strong and stable skin inflammation for about 5 days

(Figs 3 and 4). We tested the effect of deficiencies for different inflammatory genes on the kinetics of skin inflammation and on cutaneous cytokine protein levels in inflamed skin on day 14.

The role of IL-12 family cytokines in subchronic DNFB model was tested by mice deficient for IL-12/IL-23 p40-chain or IL-12/IL-35 p35-chain in comparison with C57BL/6 wild-type mice. We observed a significantly weaker skin inflammation in p35-deficient but not in p40-deficient mice. Ear thickness, cutaneous granulocyte infiltration and reaction of auricular lymph nodes were significantly reduced in p35 deficiency, exclusively (Fig. 3a and Figure S5a). Moreover, ears from p35-deficient mice exhibited a diminished immune cell infiltration and weaker vascularization in immunohistochemistry (Figure S1). Surprisingly, however, no significant changes were observed for the protein levels of TNF α , IL-1 β , IL-12p40 and IFN γ in inflamed skin (data not shown).

MyD88, originally isolated as a myeloid differentiation primary response gene, was shown to act as a critical component in the Toll-like, IL-1 and IL-18 receptor-mediated signalling cascades (20,21). In the subchronic DNFB model, a diminished skin inflammation was observed in MyD88-deficient mice with regard to ear thickness, cutaneous granulocyte infiltration and weight of corresponding lymph nodes (Fig. 3b and Figure S5b). Moreover, considerably lower protein levels of TNF α , IL-1 β , IL-12/IL-23 p40 chain and IFN γ in inflamed skin confirm the basically weaker sensitivity of MyD88 knockout mice to DNFB-induced skin inflammation.

Therapeutic treatment with prednisolone in the subchronic DNFB-induced CHS model

As assessed by ear thickness, daily prednisolone treatment from day 10 to day 13 in subchronic DNFB model led to an incremental and dose-dependent suppression of skin inflammation (Fig. 4). Thus, the treatment by 3 and 30 mg/kg prednisolone reduced the established strong ear swelling on day 10 by 46% and 81%, respectively, on day 14. Moreover, the higher prednisolone dosage decreased ear weights and cutaneous granulocyte infiltration to solvent group levels on day 14.

Discussion

We have established novel subacute and subchronic DNFB-induced mouse CHS models for T-cell-dependent inflammatory dermatoses. As most prominent in the subchronic schedule, the skin inflammation in these models exhibits hallmarks of chronic ACD and psoriasis such as pronounced keratinocyte proliferation and strong hypervascularization, immune cell infiltration and cytokine response. The subacute DNFB-induced CHS model was responsive to clinically established therapies for ACD and psoriasis such as glucocorticoids and cytokine neutralization, indicating its improved predictive character for drug discovery research. Moreover, in the subchronic model, the deficiency of IL-12/IL-35 p35 chain or of MyD88, which is involved in IL-1, IL-18, and TLR signalling, led to a significantly diminished skin inflammation. Finally, as exemplified by prednisolone therapy, the subchronic DNFB-induced CHS model permits the establishment of a stable, non-necrotic prolonged skin inflammation that allows a therapeutic treatment.

When we juxtapose our acute, subacute and subchronic DNFB-induced CHS models in C57BL/6 mice for comparison, the acute model, including two sensitizations and one challenge on the ears, elicits only a moderate inflammation with marginal immune cell

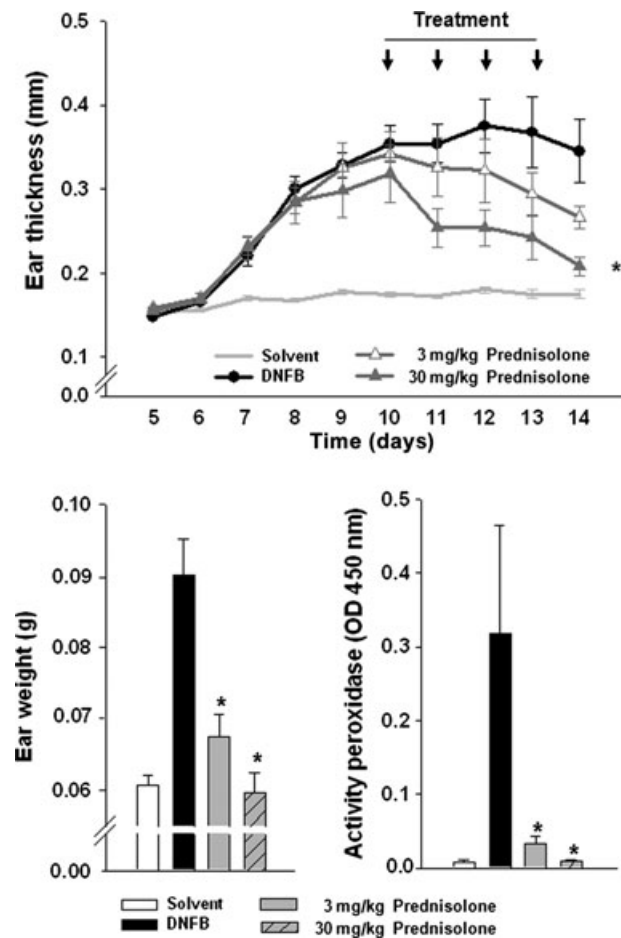


Figure 4. Prednisolone therapy in subchronic DNFB-induced mouse contact hypersensitivity model. Therapeutic oral prednisolone treatment from day 10 to day 13 dose-dependently decreased skin inflammation as assessed by ear thickness kinetics and ear weights and cutaneous myeloperoxidase activity on day 14 (female C57BL/6 mice, $n = 5$ per group). * $P < 0.05$.

infiltration, no keratinocyte proliferation and a weak vascularization. Whereas the increase in IL-1 β and IFN γ demonstrates an incipient skin inflammation, the cutaneous expression of cytokines like TNF α , IL-12 and IL-23 is not consistently induced yet. Taken together, the acute DNFB-induced CHS model lacks crucial characteristics of chronic inflammatory dermatoses like chronic ACD and psoriasis.

The DNFB-elicited inflammation in the subacute model is based on three daily challenges to initiate a strong inflammatory response, which can easily and reliably be monitored by ear thickness measuring. Compared with the acute model, it shows a more pronounced T cell infiltration, stronger vascularization, keratinocyte proliferation, an involvement of draining lymph nodes and an increase of pro-inflammatory as well as Tc1/Th1 cytokines, which are important for the immunopathophysiology of ACD and psoriasis (22,23). In our subacute DNFB-induced CHS model, TNF α , IFN γ and IL-1 β , which are strong inducers of keratinocyte proliferation (24), were highly increased at the endpoint on day 8. Moreover, IL-12/IL-35 p35 mRNA expression showed a progressive increase. In contrast, the expression of IL-23p19 was only enhanced at the endpoint on day 8 of the subacute model, indicating its role in chronic inflammation as it is also discussed for psoriasis and ACD (22,25). Taken together, the subacute DNFB-induced CHS model has several advantages in comparison with acute standard models and, because of its short time schedule and good reproducibility, is fully compatible with the requirements of early-stage drug discovery. Limitations, however, are the restriction to short-term and partially prophylactic treatment options and the mere incipient relevance of the model for inflammatory processes specific for chronic inflammation.

In our subchronic DNFB-induced CHS model, we demonstrated that prolonged daily low-dose DNFB exposure allows the formation of a stable, non-necrotic skin inflammation, displayed as a 5-day plateau of ear thickness, which is highly responsive to a therapeutic treatment by prednisolone. Moreover, the inflamed skin in the subchronic DNFB-induced CHS model exhibits a chronic ACD- and psoriasis-like skin morphology such as acanthosis, strong keratinocyte proliferation and hypervascularization, as well as a strong cutaneous immune cell infiltration, induction of cytokines and a reaction of the corresponding lymph nodes. Interestingly, exclusively in the subchronic DNFB model, we observed a strong up-regulation not only of the Th1/Tc1 cytokines and T-bet expression but also of Th2 cytokines.

This increase of the cutaneous Th2 response in the subchronic DNFB model is in accordance with data from Kitagaki et al. (26,27) showing a cutaneous and lymph node Th1/Tc1 to Th2 shift in repeated CHS responses using TNCB. Interestingly, for human ACD, Borg et al. (28) described a predominantly Th2 *in vitro* response to nickel in nickel-allergic individuals. Moreover, a very recent publication hints to a Th2 shift also in patients with chronic plaque psoriasis (29).

When assessing the likely predictive value of animal models for a disease, an adequate response to clinically established therapies is pivotal. Treatment by glucocorticoids is the standard treatment for ACD and is strongly effective in our subacute and subchronic DNFB-induced mouse CHS models.

In psoriasis, strategies for therapeutic intervention into the cytokine network currently include the antagonization of TNF α ,

IL-12 and IL-23 and IL-18 by neutralizing biologics (17,30). Several TNF α blockers (etanercept, adalimumab and infliximab) and ustekinumab, a monoclonal antibody targeting the common p40 subunit of IL-12 and IL-23, are approved for psoriasis therapy (31). However, the situation for ACD is not clear so far. Whereas Cassano et al. (32) found a good clinical response to infliximab in a patient with atopic dermatitis and a concomitant ACD, Agnusti et al. (33) reported an incomplete response to etanercept in a patient with psoriasis, which was likely due to a chronic ACD. Moreover, very recently, Bangsgaard et al. (34) described an almost completely missing effect of IL-12/IL-23 neutralization by ustekinumab in five patients with chronic ACD. Taken together, there are no clear clinical data demonstrating an efficacy of TNF α and IL-12/IL-23 neutralization in chronic ACD.

Keeping this in mind, it might be not surprising that we observed only moderate effects of these approaches in our subacute DNFB-induced CHS model. Administration of neutralizing antibodies to TNF α and to the IL-12/IL-23 p40-chain led to a significant but modest reduction in skin inflammation as assessed by ear thickness. Comparable data were also achieved for the neutralization of IL-18 in the subacute DNFB model.

In the subchronic DNFB-induced CHS model, we characterized the impact of IL-12 family cytokines on skin inflammation by using mice deficient for the IL-12/IL-23 p40-chain or the IL-12/IL-35 p35 chain. p40 knockout mice are described to be defective in Th1 and (partly) in Th17 cytokine responses because of the missing activities of IL-12 and IL-23 (35). Interestingly, however, p40-deficient mice are reported to demonstrate a normal, rather than a decreased CHS response in an acute DNFB-induced mouse model (36). This might be explained by the high redundancy of cytokine activities in CHS (37) and by pro-inflammatory effects because of the loss of anti-inflammatory p40 homodimers (38).

In our subchronic model, p40 knockout mice did not exhibit a reduction in the inflammatory response to DNFB, neither in incipient nor in prolonged skin inflammation. In contrast, p35-deficient mice showed a basically weaker skin inflammation with less-immune cell infiltration than wild-type mice. Surprisingly, besides a non-significant decrease in IL-12p40 protein (data not shown), which is likely due to the loss of the IL-12 heterodimer, no significant changes in comparison with wild-type mice were observed for other important cytokines such as TNF α , IL-1 β and IFN γ (data not shown). From these data, the mechanisms leading to the diminished skin inflammation in p35-deficient mice are not clear for us.

Furthermore, we used mice deficient for MyD88 to characterize the impact of IL-1 family cytokine and TLR signalling in the subchronic DNFB model (20,21). MyD88 knockout mice showed a moderately diminished skin inflammation. The cutaneous cytokine levels on day 14, however, were profoundly diminished in comparison with those in wild-type mice.

Our data indicate an importance of MyD88 signalling especially for the establishment of a strong cytokine response in CHS. In fact, a crucial role of the danger and proinflammatory signalling through the IL-1 β - and IL-18-generating inflammasome, Toll-like receptors and MyD88 have been described for the initiation of CHS responses (4,39–41). These pathways, however, might also be involved into the CHS elicitation phase. So, the treatment with an anti-IL-18 antibody before the first challenge led to a significant inhibition of ear thickness increase in our subacute DNFB model.

In summary of these data, the repeated DNFB challenges in our subacute and subchronic models generate a skin inflammation that is sensitive to the inhibition of MyD88-mediated danger and inflammatory cytokine signalling as well as to the antagonization of cytokines, which are pathophysiologically relevant for psoriasis and likely also for ACD.

Taken together, the newly established subacute and subchronic DNFB-induced mouse CHS models better than the acute DNFB model reflect the morphological and functional specifics of chronic inflammatory skin diseases like chronic ACD and psoriasis. This presumably leads to an improved predictive value of these models for the profiling of novel therapeutic approaches. Moreover, the subchronic DNFB-induced CHS model allows a purely therapeutic treatment of a stable pre-established inflammation according to the clinical situation. Overall, these mouse models could be instrumental in characterizing immunological

mechanisms of ACD and psoriasis, and they provide well-defined advantages for the characterization of novel drug candidates for the treatment of T-cell-dependent chronic inflammatory dermatoses.

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Conflict of interest

All authors are employees and, partially, stockholders of Bayer Pharma AG.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunohistochemical characterization of DNFB-induced mouse CHS models.

Figure S2. Comparison of inflammation parameters in DNFB-induced mouse CHS models.

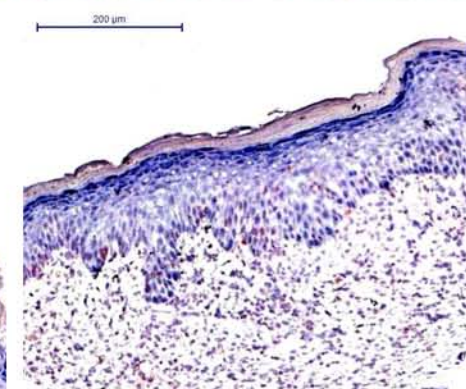
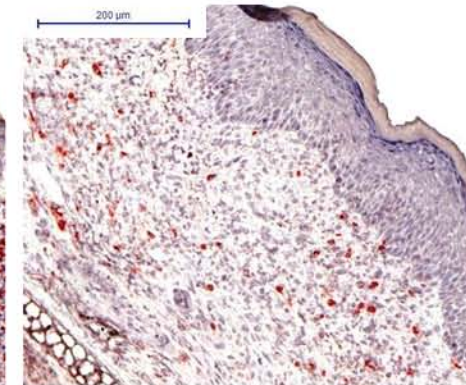
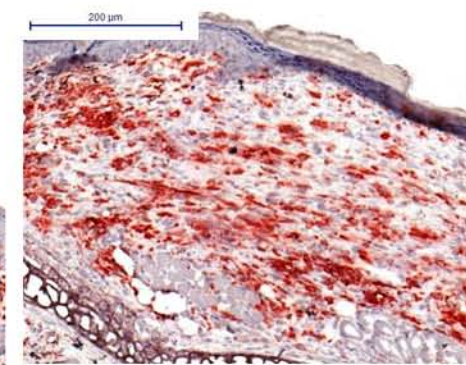
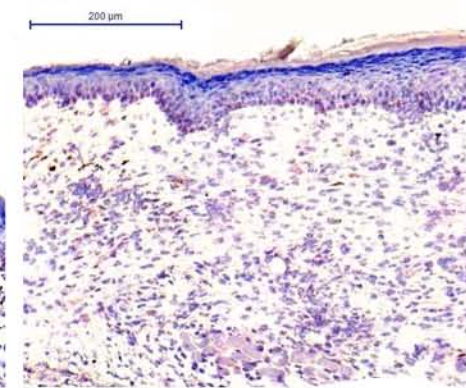
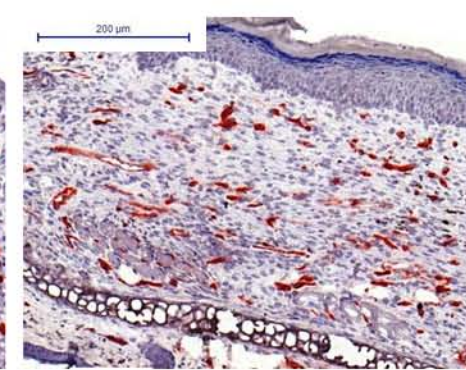
Figure S3. Cytokine gene expression in subchronic DNFB-induced mouse CHS model.

Figure S4. Kinetics of inflammation parameters in subacute DNFB-induced mouse CHS model and subacute DNFB-induced CHS model in nude mice.

Figure S5. Subchronic DNFB-induced CHS model in gene-deficient mice.

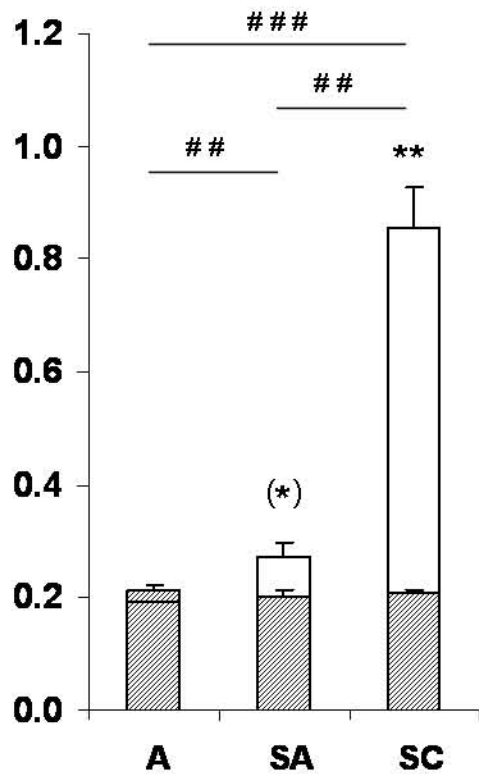
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Chronic Model
IL-12/IL-35 p35^{-/-}

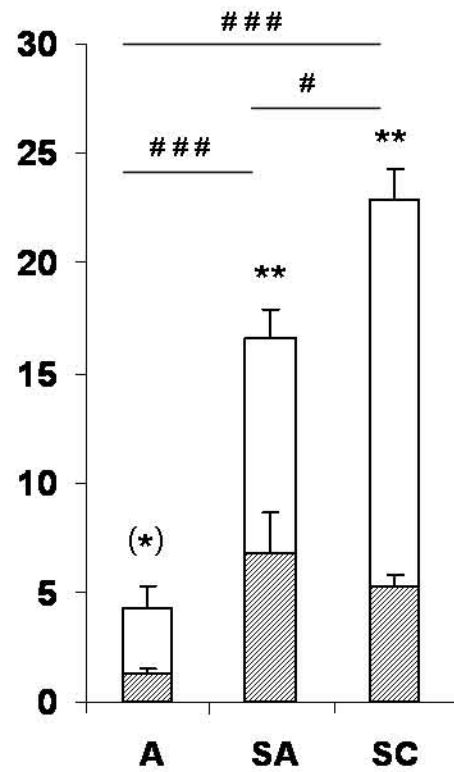
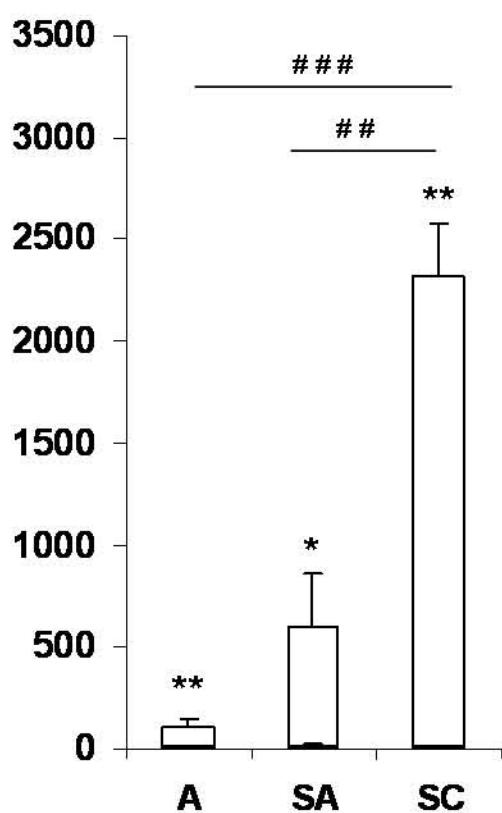
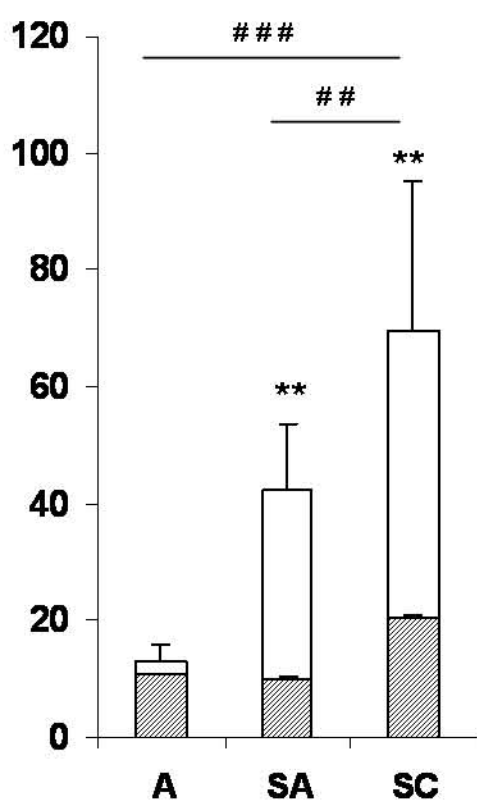
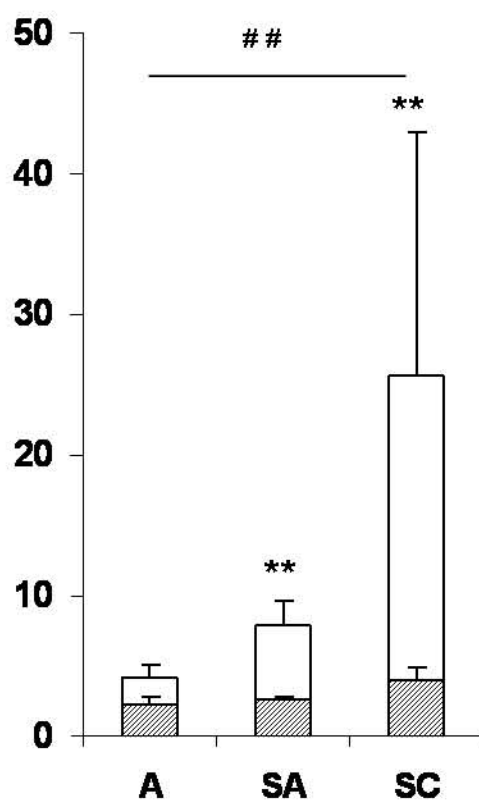
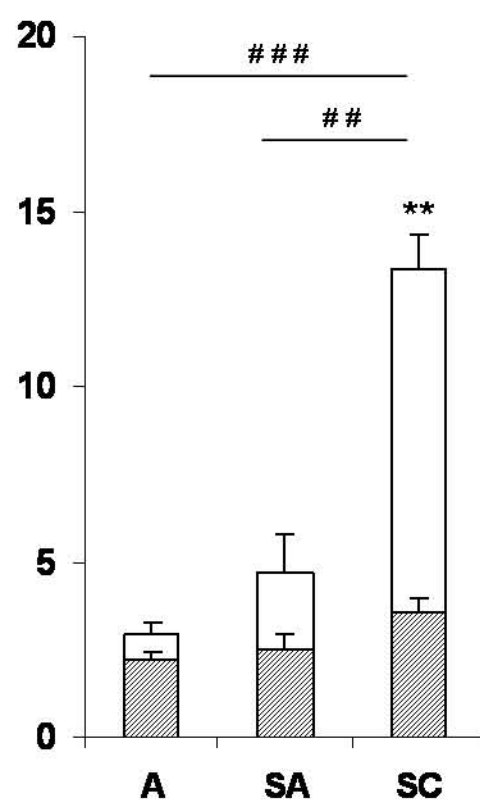


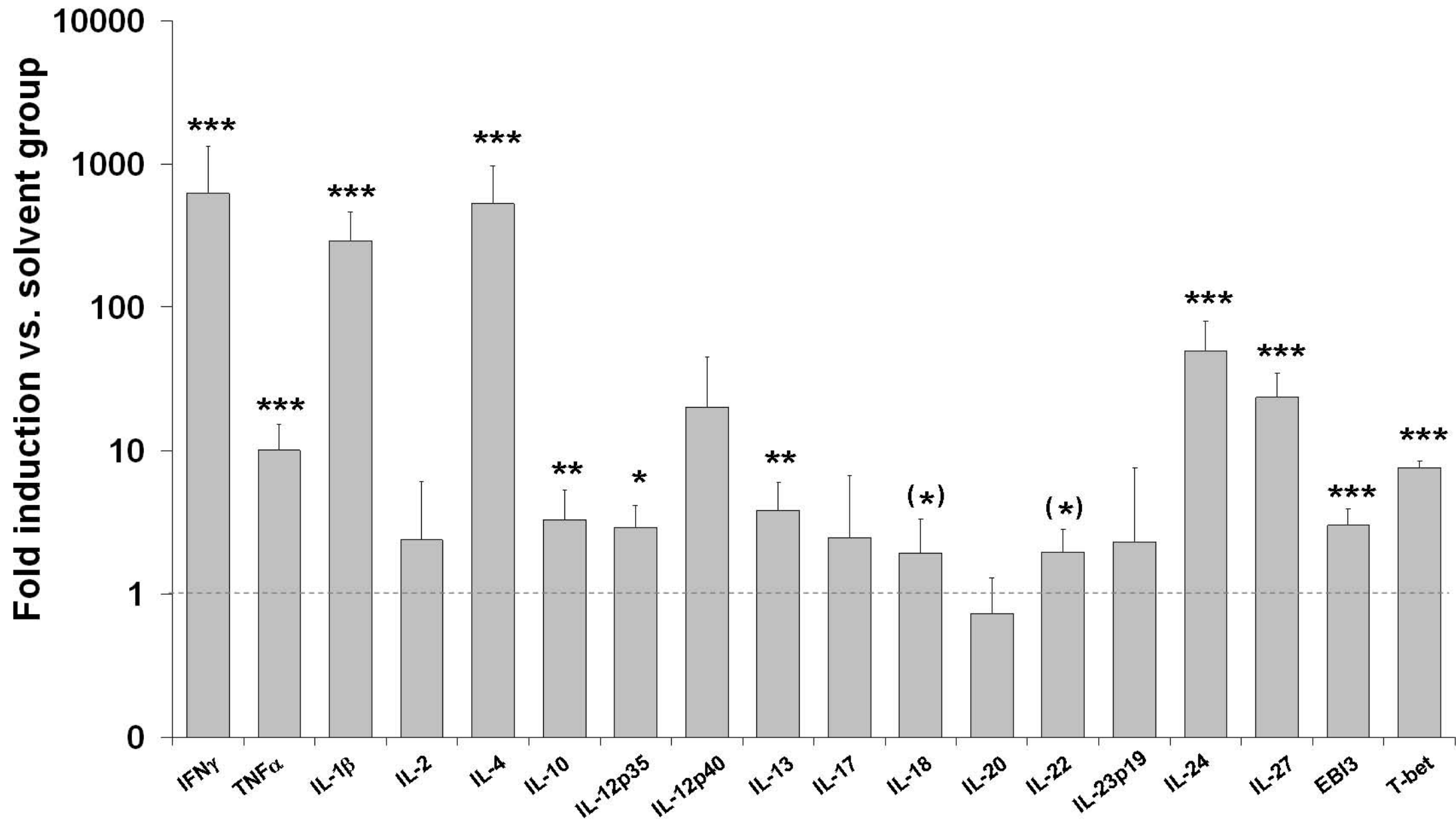
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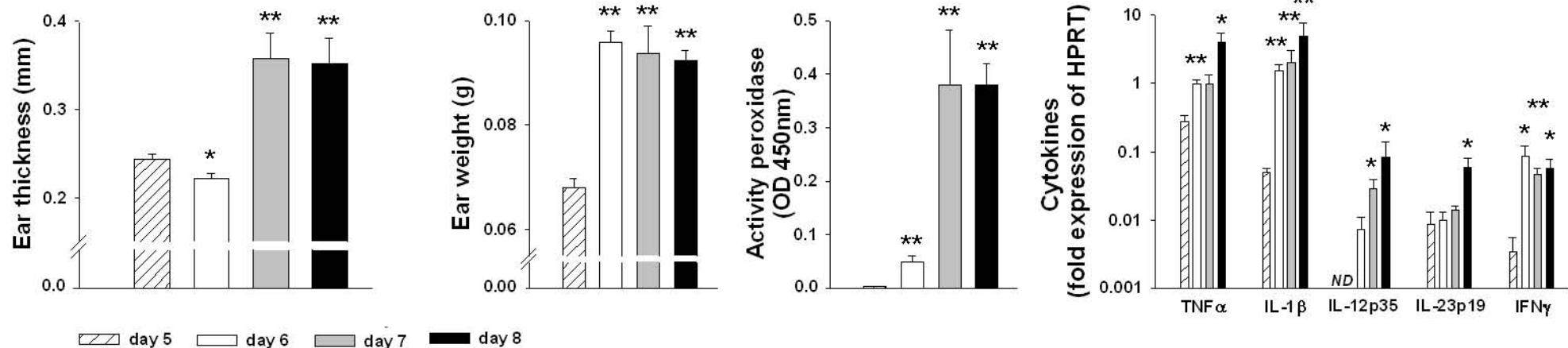
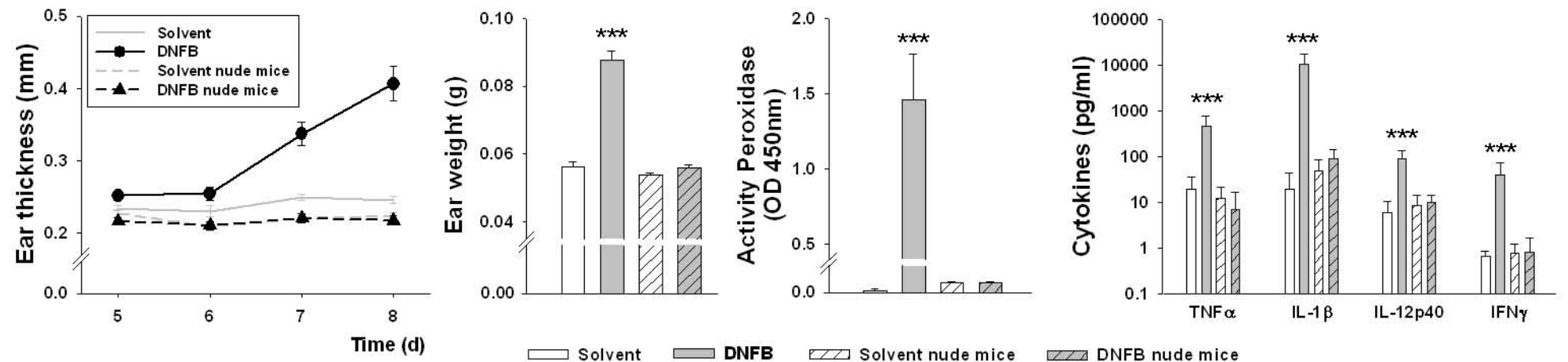
Ear thickness (mm)

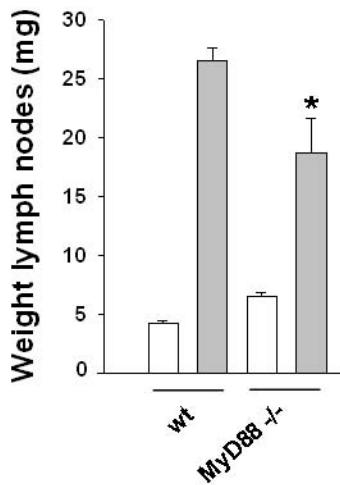
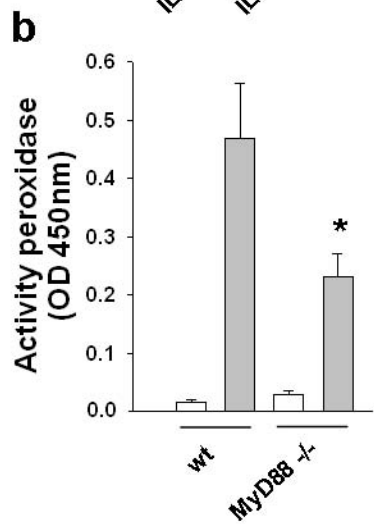
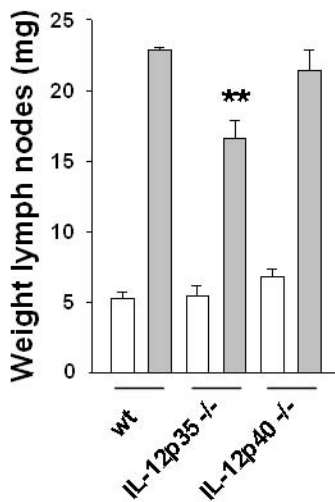
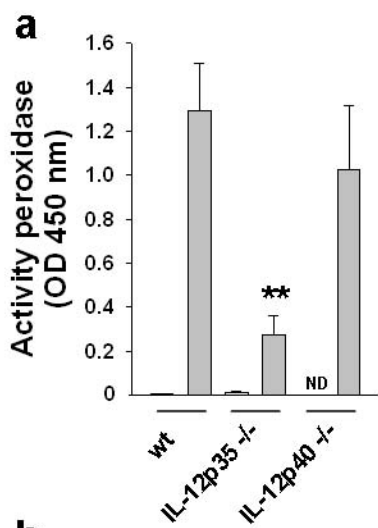


Weight lymph nodes (mg)

**b**IL-1 β (pg ml⁻¹)IL-10 (pg ml⁻¹)IL-2 (pg ml⁻¹)IL-5 (pg ml⁻¹) Solvent DNFB



a**b**



□ Solvent ■ DNFB

Figure S1. Immunohistochemical characterization of DNFB-induced mouse CHS models. Skin derived from different DNFB-induced ear inflammation models in C57BL/6 wildtype mice as well as acute solvent control were stained by antibodies against CD31, Ki67, MHCII (I-Ad), CD4 and CD8. Increasing cutaneous infiltration with MHCII⁺ cells as well as with CD4⁺ helper and CD8⁺ cytotoxic T cells is seen with model prolongation. Moreover, only the subchronic DNFB-induced CHS model clearly shows characteristics of chronic eczema such as acanthosis, strong hypervascularization (CD31 staining) and pronounced keratinocyte proliferation (Ki67 staining). In skin derived from C57BL/6 mice deficient for the IL 12/IL 35 p35-chain, a basically weaker inflammation was established after performing the subchronic DNFB-induced CHS model (right column).

Figure S2. Comparison of inflammation parameters in DNFB-induced mouse CHS models.

(a) Ear thickness and weight of auricular lymph nodes as well as (b) cytokine levels in ear homogenates were determined at the endpoints in acute (A) (d6, n=6), subacute (SA) (d8, n=7) and subchronic (SC) (d14, n=10) DNFB models in female C57BL/6 mice. (*) P<0.1, * P<0.05, ** P<0.01 vs. solvent group; # P<0.05, ## P<0.01, ### P<0.001 comparison of DNFB groups between the three models.

Figure S3. Cytokine gene expression in subchronic DNFB-induced mouse CHS models. Relative cytokine mRNA expression in lesional skin from DNFB group (n=10) in comparison to solvent group (n=6) on day 14 of subchronic DNFB-induced CHS model. (*) P<0.1, * P<0.05, ** P<0.01 vs. solvent group.

Figure S4. Kinetics of inflammation parameters in subacute DNFB-induced mouse CHS model (a) Kinetics of ear thickness, ear weight, myeloperoxidase activity and cytokine mRNA expression in ear homogenates has been determined in subacute DNFB model (n=5). ND – not detected, * P<0.05, ** P<0.01 vs. day 5 (pre-challenge). (b) To characterize T cell dependence of subacute DNFB model, kinetics of ear thickness as well as ear weights and myeloperoxidase activity and cytokine levels in ear homogenates on day 8 have been compared for male C57BL/6 wildtype and nu/nu mice (n=13). ND – not detected, *** P<0.001 vs. respective solvent group.

Figure S5. Subchronic DNFB-induced CHS model in gene-deficient mice (a) Deficiency for IL 12/IL 35 p35-chain but not for IL 12/IL 23 p40-chain inhibited skin inflammation in female C57BL/6 mice (wildtype n=10, p35-deficient n=12, p40-deficient n=9) as assessed by cutaneous myeloperoxidase activity and auricular lymph node weights on day 14. (b) Skin inflammation in subchronic DNFB-induced CHS model was diminished in female MyD88-deficient in comparison to wildtype C57BL/6 mice. Cutaneous myeloperoxidase activity and auricular lymph node weights on day 14 were determined in female C57BL/6 wildtype (n=6) and MyD88-deficient mice (n=8). * P<0.05, ** P<0.01 vs. DNFB group in wildtype mice.