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Inhibition of Allergic Contact Dermatitis to DNCB But Not to Oxazolone in Interleukin-4-Deficient Mice

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The role of interleukin-4 as a regulator of immune responses in the skin is investigated with regard to the outcome of contact hypersensitivity reaction in interleukin-4-deficient BALB/C mice. In previous studies conflicting results were obtained concerning the role of interleukin-4 in contact hypersensitivity reactions supporting either a proinflammatory or rather an inhibitory function of this cytokine. Interleukin-4 deficient BALB/C mice sensitized to 2,4-dinitrochlorobenzene showed after challenge a significant reduction in magnitude and duration of the contact hypersensitivity response in comparison with wild-type mice. This attenuation was accompanied by a significant reduction of edema and cellular infiltrates in the dermis and a lacking induction of IL-10 mRNA expression in skin. Also, adoptive transfer experiments revealed that BALB/C mice failed to exhibit contact hypersensitivity after injection of lymph node cells obtained from sensitized interleukin-4 deficient mice. To examine further the role of the contact allergen used to induce the contact hypersensitivity response, mice were also sensitized and challenged with Oxazolone. Here a similar magnitude and duration of contact hypersensitivity in both the interleukin-4 deficient mice and BALB/C control mice was observed. This indicates that the contact hypersensitivity response to 2,4-dinitrochlorobenzene and Oxazolone may partly evolve on different pathways being dependent and independent of interleukin-4. Our results clearly show that the complete loss of endogenous interleukin-4 expression in BALB/C mice is associated with an impaired manifestation of contact hypersensitivity response to 2,4-dinitrochlorobenzene, implying an important proinflammatory function of this cytokine.
Key words: contact hypersensitivity/cytokine deficiency/skin/transgenic mice.

Despite considerable progress in elucidating the nature of cellular interactions involved in contact hypersensitivity (CHS) reactions neither the role of particular cytokines nor the role of T cell subsets ($CD8^+$ and $CD4^+$ T cells) are fully understood. *In vivo* and *in vitro* antibody depletion studies from several laboratories indicated $CD4^+$ T cells as the effector T cells in CHS whereas others observed the ability of $CD8^+$ T cells to mediate CHS (Miller and Jenkins, 1985; Gosinski and Tigelaar, 1990; Hauser, 1990; Gautam *et al*, 1991; Xu *et al*, 1996). The CHS response and delayed-type hypersensitivity reaction are often regarded as Th1-mediated processes, as it could be shown that T-helper 1 cells [interleukin (IL)-2, interferon-γ producers] could passively transfer these reactions (Cher and Mosmann, 1987; Dieli *et al*, 1994). Studies aimed at defining the role of T-helper 2 cells (IL-4, IL-10 producer) provided evidence that these cells are inactive or may even exert suppressive effects in CHS reactions (Cher and Mosman, 1987; Asada *et al*, 1997). Th2 cells, however, have also been shown to be critically involved in inflammatory processes as, e.g., chronic graft-versus-host reactions, autoimmune diseases and atopic dermatitis (Fanslow *et al*, 1991; Goldman *et al*, 1991; Van der Heijden *et al*, 1991; Erb *et al*, 1997).

IL-4 is a pleiotropic cytokine product of Th2 cells regulating immunoglobulin isotype switching (IgE, IgG4). Arguably, the most important effect of IL-4 is its role in T cell growth, especially the development of Th2 lineage. It harbors B-cell stimulating activities and can suppress Th1-mediated immune responses (Mosmann and Coffman, 1989; Mueller *et al*, 1997). It is well known that IL-4 is produced in murine skin during the elicitation phase of CHS (Asada *et al*, 1997), but whether it plays a proinflammatory or anti-inflammatory part remains unclear. IL-4 has been reported to exert negative regulatory effects on the CHS response as the injection of recombinant IL-4 reduced the magnitude of the challenge response to the hapten (Gautam *et al*, 1992). Furthermore, pretreatment with monoclonal anti-IL-4 antibodies before challenge led to increased ear swelling compared with mice pretreated with isotype control monoclonal antibodies (Asada *et al*, 1997). Proinflammatory activities of IL-4, however, were suggested from experiments by Salerno *et al* (1995) using anti-IL-4 antibodies, which markedly inhibited the manifestation of contact dermatitis (CD) and the passive transfer of the CHS reaction by immune lymph node cells.

Transgenic mouse technology has enormously advanced the opportunities to study the role of a specific cytokine in CD as the role of genes suspected to be involved in the pathogenesis of CD can now be studied in mice characterized by an overexpression or a loss of function of a particular gene. The IL-4-deficient mice, generated by Kühn *et al* (1991), show normal T cell and B cell development, but strongly reduced levels of IgG1 and IgE.

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Abbreviations: CHS, contact hypersensitivity; CD, contact dermatitis; DNBS, 2,4-dinitrobenzoic acid; DNCB, 2,4-dinitrochlorobenzene.

Moreover, independently generated IL-4-deficient mice (Kopf *et al*, 1993) indicated a requirement of IL-4 in the generation of Th2-derived cytokines and an impairment of immune responses dependent on these cytokines.

In order to define better the role of endogenous IL-4 in inducing and regulating CHS we treated BALB/C mice lacking the IL-4 gene with two obligate contact sensitizing agents [2,4-dinitrochlorobenzene (DNCB), Oxazolone] to investigate the CHS response in the absence of IL-4.

MATERIALS AND METHODS

Mice Breeding pairs of the IL-4-deficient BALB/C mice were a kind gift from Dr. W. Müller, Institute of Genetics, University of Cologne, Germany. They were used in the tenth back-cross generation. Wild-type littermates were purchased from Boongart, Sweden. Mice were bred and kept in specific pathogen free status under standard diet. They were used at 8–16 wk of age.

Induction and challenge of CHS Mice were sensitized topically with 100 μl of a 5% DNCB in an olive oil, acetone solution (1:4) on the ventral unshaved abdomen. Five days later, 10 μl of a 1% DNCB olive oil, acetone solution were each applied to both sides of the right ear. The left ear was exposed to vehicle alone. For CHS to Oxazolone, the same concentrations and vehicle was used for sensitization and challenge.

Measurements of ear thickness to determine the cutaneous manifestation of CHS was performed by using a micrometer (Oditest, Kroeplin). The area below the apex of the ear was measured leaving a barely perceptible margin of ear visible above the edge of the measuring device. Ear swelling was calculated by subtracting the prechallenge value from the postchallenge value (Gad *et al*, 1986).

Histology For histologic examination mice were killed 24 h after challenge. Ears were fixed in formalin (10%) for 1–3 d and processed for paraffin embedding. Tissue sections (6 μm) were stained with hematoxylin and eosin, mounted, and analyzed in a blinded fashion by two independent observers.

Immunohistochemical staining Ears were obtained 12, 24, and 72 h after challenge with DNCB. Ears were snap frozen in liquid nitrogen. Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining of cryosections was performed following established procedures. Rabbit anti-mouse immunoglobulins and APAAP complexes were obtained from Dako (Hamburg, Germany). The alkaline phosphatase reaction was demonstrated by incubation in a solution containing Fast Red TR (1 mg per ml) and naphtol AS-TR phosphate (0.2 mg per ml) (Sigma, St. Louis, MO). Levamisole (0.24 mg per ml) was added to block endogenous alkaline phosphatase activity. Monoclonal anti-mouse antibodies to ICAM-1, and IA (major histocompatibility complex class II) were obtained from Dianova (Hamburg, Germany). Negative controls consisted of the omission of primary antibodies and staining with the dye alone. As a positive control a polyclonal antibody directed against keratin was used. The stained sections were reviewed by two independent observers.

Reagents and culture media T cell proliferation assays were performed in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% antibiotic/antimycotic (Gibco, Karlsruhe, Germany), 10 mM HEPES buffer solution, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol. For fluorescence-activated cell sorting (FACS) analysis, the monoclonal antibody anti-$CD4^+$ (L3T4), anti-$CD8^+$ were used, and anti-rat IgG2aκ (all purchased from PharMingen, San Diego, CA). For magnetic cell sorting isolation a puffer with 2 mM EDTA and 0.5% bovine serum albumin (Sigma) was used. Fluorescence-activated cell sorter analysis was performed to confirm the purity of isolated $CD4^+$ and $CD8^+$ T cells. After magnetic cell sorting T cells were stained with anti-$CD4^+$ (L3T4), anti-$CD8^+$, and anti-rat IgG2aκ as isotype control (all purchased from PharMingen) following the instructions of the manufacturer. $CD4^+$ T cells were 97% and $CD8^+$ T cells were 80% pure (data not shown).

Preparation of antigen-presenting cells from the spleen Inguinal, axillary, and brachial lymph nodes were obtained 5 d after *in vivo* sensitization. Single cell suspensions from lymph nodes were purified for $CD4^+$ and $CD8^+$ T cells by magnetic cell sorting as described by the supplier (Milteny Biotec, GmbH Bergisch-Gladbach, Germany). $CD4^+$ T cells were plated at 10^5 cells per well in the presence of 5×10^5 irradiated (3000 RAD) and 2,4-dinitrobenzoic acid (DNBS)-modified spleen cells as described by Shearer (1974). T cell lines were restimulated (six to eight times) by adding fresh DNBS-modified irradiated syngeneic stimulator cells every 10–12 d. T cell proliferation was determined by the incorporation of [^{3}H]thymidine the last 17 h of culture. Results are presented as the mean of assays performed in triplicate.

Table I. Primers for RT-PCR were designed by using sequences obtained from Genbank

GAPDH 5′: 5′ TCA TGA CCA CAG TCC ATG CCA TCA C 3′
GAPDH 3′: 5′ GCC GTA TTA ATT GTC ATA CCA GGA AAT GA 3′
IL-10 5′: 5′ ATG CAG GAC TTT AAG GGT TAC TTG GGT T 3′
IL-10 3′: 5′ AAT TCG GAG AGA GGT ACA AAC GAG GTT T 3′
IL-4 5′: 5′ ATG GGT CTC AAC CAG CTA GT 3′
IL-4 3′: 5′ GCT CTT TAG GCT TTC CAG GAA GTC 3′

Enzyme-linked immunosorbent assay Cytokine-specific sandwich enzyme-linked immunosorbent assay for determining quantities of IL-1β, IL-2, IL-4, interferon-γ, and IL-10 in the supernatants from $CD4^+$ and $CD8^+$ cell lines were performed generally following the instructions of the supplier (Genzyme, Cambridge, MA).

Adoptive transfer experiments Cell suspensions obtained from lymph nodes of DNCB-sensitized mice were injected into the ears of naïve mice (five mice per group) (2×10^5 per 20 μl phosphate-buffered saline) as described previously (Dieli *et al*, 1994). The mice were immediately challenged by applying 10 μl 1% DNCB in acetone:olive-oil (4:1) on both sides of the right ear. In these experiments negative controls were groups of mice injected with phosphate-buffered saline, s.c., into the ears and challenged with 1% DNCB as described above. Ear thickness was measured shortly before challenge and swelling was assessed 12 and 24 h after challenge with the use of an engineer's micrometer (Kroeplin, Schluechtern, Germany) as described.

Reverse transcriptase-polymerase chain reaction (PCR) for IL-4 and IL-10 Total RNA was isolated by the guanidium/thiocyanate/caesium chloride method. Reverse transcription was performed according to the manufacturers protocol (Gibco, BRL) using oligo d(T)16 primer. Primers were synthesized by MWG (Ebersberg, Germany) as shown in **Table I**.

The cycling conditions were as follows: denaturation for 1 min at 94°C, annealing for 1.5 min at 55°C (IL-4, IL-10) and 57°C (GAPDH). Primers, mRNA concentrations, and PCR cycles were titrated to establish standard curves to demonstrate linearity that allowed semiquantitative analysis of signal strength. IL-4 and IL-10 transfected Hy 358 cells were used as positive controls (kindly provided by Dr. W. Müller).

After PCR, samples were electrophoresed on agarose gels, blotted and hybridized to P^{32} end-labeled oligos (IL-4: 5′ AAA ATA TGC GAA GCA CCT TGG AAG CCC TAC 3′; IL-10 5′ GCC TTG TAG ACA CCT TGG TCT TCC AGC TTA 3′) or a P^{32} labeled random primed GAPDH cDNA probe (kindly provided by Dr. J. Uitto, Thomas Jefferson University, Philadelphia, PA). After hybridization filters were washed and exposed at −80°C to a radiosensitive filter.

Statistics The double-tailed Student's t-test was used to compare the significance of differences between groups.

RESULTS

Impaired and shortened CHS response to DNCB in the absence of endogenous IL-4 To evaluate the capacity of IL-4-deficient mice to develop CHS, 5% DNCB were used for sensitization on the abdomen and 1% DNCB for challenge on the right ear 5 d later. The left ear was exposed to the vehicle (acetone/olive oil) alone. As shown in **Fig 1** the time courses of CHS responses were similar in IL-4-deficient mice and control mice. Maximum of ear swelling in both mouse strains was observed after 24 h. Notably, ear swelling responses of IL-4-deficient mice were strongly reduced which exhibited a 40%–60% reduction of ear swelling after 12 h ($p < 0.01$) compared with control mice. The strongest suppression of CHS in IL-4-deficient mice was observed 12–24 h after challenge with DNCB (**Fig 1**). After 24 h ear thickness declined in both IL-4-deficient mice and control mice. Mutant mice showed an ear thickness that was back to baseline after 8 d whereas the ear thickness of control mice returned to normal size with a delay of 10 d after challenge.

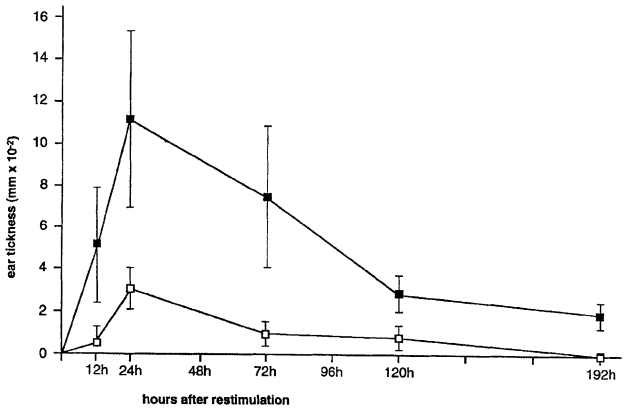

Figure 1. Attenuated CHS to DNCB in IL-4-deficient mice. IL-4-deficient mice and wild-type mice were sensitized with 1% DNCB on day 0 and challenged on the right ear on day 5 with 1% DNCB. The left ear was treated with vehicle acetone/olive oil alone. The increase of ear thickness was measured 12 h to 8 d after challenge. Data represent mean swelling values obtained with six mice per group and are representative of four independent experiments (statistical significance: $p < 0.005$) ■, wild-type mice; □, IL-4-deficient mice.

Histologic examination of the challenge and control ears of the wild-type and IL-4-deficient mice after 24 h revealed a reduced edema and cellular infiltrate in the mutant mice confirming the results of the mouse ear swelling test (**Fig 2*a–d***). Furthermore, ICAM-1 and IA (major histocompatibility complex class II) molecule expression as determined by immunohistochemical staining also was markedly reduced after 12 and 24 h in the mutant mice (**Fig 2*e–j***).

In heterozygous IL-4 BALB/C mice (IL-4 +/–) CHS response to DNCB is similar as in wild-type BALB/C mice Differing results were recently reported using IL-4-deficient mice on the genetic background C57BL/6 (Berg *et al*, 1995; Weigman *et al*, 1997). In order to exclude influences of the genetic background on the diminished CHS response, IL-4-deficient mice were backcrossed to wild-type BALB/C mice in order to generate heterozygous IL-4 (+/–) BALB/C mice. When these heterozygous mice were sensitized and challenged as described above no significant difference in ear swelling response compared with wild-type mice was observed in two experiments performed with five mice per group (data not shown).

Lymph node cells from IL-4-deficient mice fail to induce CHS in BALB/C mice We analyzed the ability of lymph node cells of sensitized mice to transfer CHS. Cells were injected subcutaneously into the ears of naïve mice followed by challenge with DNCB applied epicutaneously. IL-4-deficient mice were sensitized with 5% DNCB on the abdomen, 5 d later axillary and inguinal lymph nodes were taken and processed to single cell suspensions. Lymph node cells (2×10^5) were injected subcutaneously into the ears of naïve BALB/C mice. The same experiment was performed with lymph node cells of BALB/C mice, which were injected in IL-4-deficient mice as described above. Challenge was performed immediately after injection of lymph node cells by applying 1% DNCB on both sides of the ear. Ear swelling was measured 12 and 24 h after challenge. As expressed in **Fig 3** BALB/C mice failed to exhibit CHS after injection of lymph node cells of sensitized IL-4-deficient mice. On the other hand IL-4-deficient mice showed a normal CHS response to DNCB after injection of lymph node cells of sensitized BALB/C mice.

The CHS response to oxazolone is not impaired in IL-4-deficient mice To evaluate the CHS reaction to oxazolone, a less potent allergen inducing both a T cell response and immunoglobulin response, we sensitized IL-4-deficient (IL-4 –/–) and BALB/C wild-type mice (IL-4 +/+) with 5% oxazolone. Five days later, mice were challenged with 1% oxazolone and ear swelling was measured as described above. Although mutant mice showed a reduced CHS response in comparison to wild-type BALB/C mice the difference was not significant (**Fig 4**). These results are in agreement with previous investigations showing a normal CHS reaction in IL-4-deficient C57BL/6 mice to Oxazolone (Berg *et al*, 1995).

IL-10 mRNA is not induced during the CHS response to DNCB in IL-4-deficient BALB/C mice but in CHS response to oxazolone In order to confirm that IL-4 is adequately induced under the experimental conditions used, semiquantitative reverse transcriptase-PCR for IL-4 was performed. After sensitization with 5% DNCB mice were challenged with 1% DNCB on the right ear. The left ear was exposed to vehicle alone. Twenty-four hours after challenge mice were killed and ears taken for RNA extraction. As shown in **Fig 5(*A*)** (*lane 2*) there is a strong induction of IL-4 mRNA in wild-type mice after 24 h. As expected IL-4 mRNA induction in IL-4-deficient mice (IL-4 –/–) is lacking (**Fig 5*A***, *lane 4*).

IL-10 has previously been shown to be involved in the natural negative control of CHS responses in the skin (Enk *et al*, 1993). Recent studies have shown that after application of an allergen, IL-10 mRNA is rapidly upregulated with a peak at 12 h returning to normal levels thereafter (Ferguson *et al*, 1994). Therefore, IL-10 mRNA levels were determined to investigate the role of IL-10 expression in the attenuated CHS response of IL-4-deficient mice. Interestingly, IL-10 mRNA levels were strongly induced after 12 h only in the wild-type mice (**Fig 5*C***, *lane 2*), whereas no such induction could be detected in the mutant mice (**Fig 5*C***, *lane 4*) indicating that local up-regulation of IL-10 is not responsible for the reduced CHS response in IL-4-deficient mice. In contrast 12 h after challenge to oxazolone induction of IL-10 mRNA was observed both in control and IL-4-deficient mice (**Fig 5*E***, *lanes 2* and *4*).

Normal proliferative capacity of $CD4^+$ and $CD8^+$ T cells in IL-4-deficient mice In order to evaluate whether an altered proliferation behavior of $CD4^+$ and $CD8^+$ T cells plays a part in the reduced CHS response, T cell proliferation assays were performed. *In vivo* 5% DNCB-sensitized mice were killed and reactive axillary and inguinal lymph nodes were obtained. Single cell suspensions from these lymph nodes were separated for $CD4^+$ and $CD8^+$ T cells by magnetic cell sorting. Pure $CD4^+$ and $CD8^+$ T cells controlled by fluorescence-activated cell sorter were incubated with syngeneic irradiated and DNBS-modified spleen cells. Proliferation activity was measured by the incorporation of $[^3H]$thymidine the last 17 h of culture. Proliferation indices of $CD4^+$ and $CD8^+$ T cells from IL-4 (+/+) and IL-4 (–/–) mice were similar. $CD4^+$ and $CD8^+$ T cell lines derived from IL-4 (+/+) and IL-4 (–/–) mice were highly specific for the antigen DNBS indicating that IL-4-deficient mice show normal antigen specific T cell proliferation (data not shown).

Differential induction of IL-10 synthesis by immune $CD4^+$ and $CD8^+$ T cells of IL-4 (–/–) and IL-4 (+/+) mice after hapten sensitization As the results of ear swelling tests showed an attenuation of the CHS response in IL-4-deficient mice and antigen-specific proliferation capacity of T cells was unchanged, hapten-immune $CD4^+$ and $CD8^+$ T cells were stimulated by culture with syngeneic DNBS modified spleen cells. Cytokine profiles produced by $CD4^+$ and $CD8^+$ T cells from *in vivo* DNCB-sensitized mice were analyzed from the culture supernatants of the cells. IL-4 production was not observed in $CD4^+$ IL-4 (–/–) T cells, as predicted, whereas IL-4 production of control $CD4^+$ T cells reached a maximum 12 h after *in vitro* restimulation with DNBS. Forty-eight hours after stimulation the IL-4 concentration declined and went to nondetectable levels after 144 h (**Fig 6*b***). IL-2 production of $CD4^+$ T cells from both mouse strains were similar as were IL-1α and IL-1β concentrations (data not shown). Analysis of the cytokine production of $CD8^+$ cells revealed, however, that

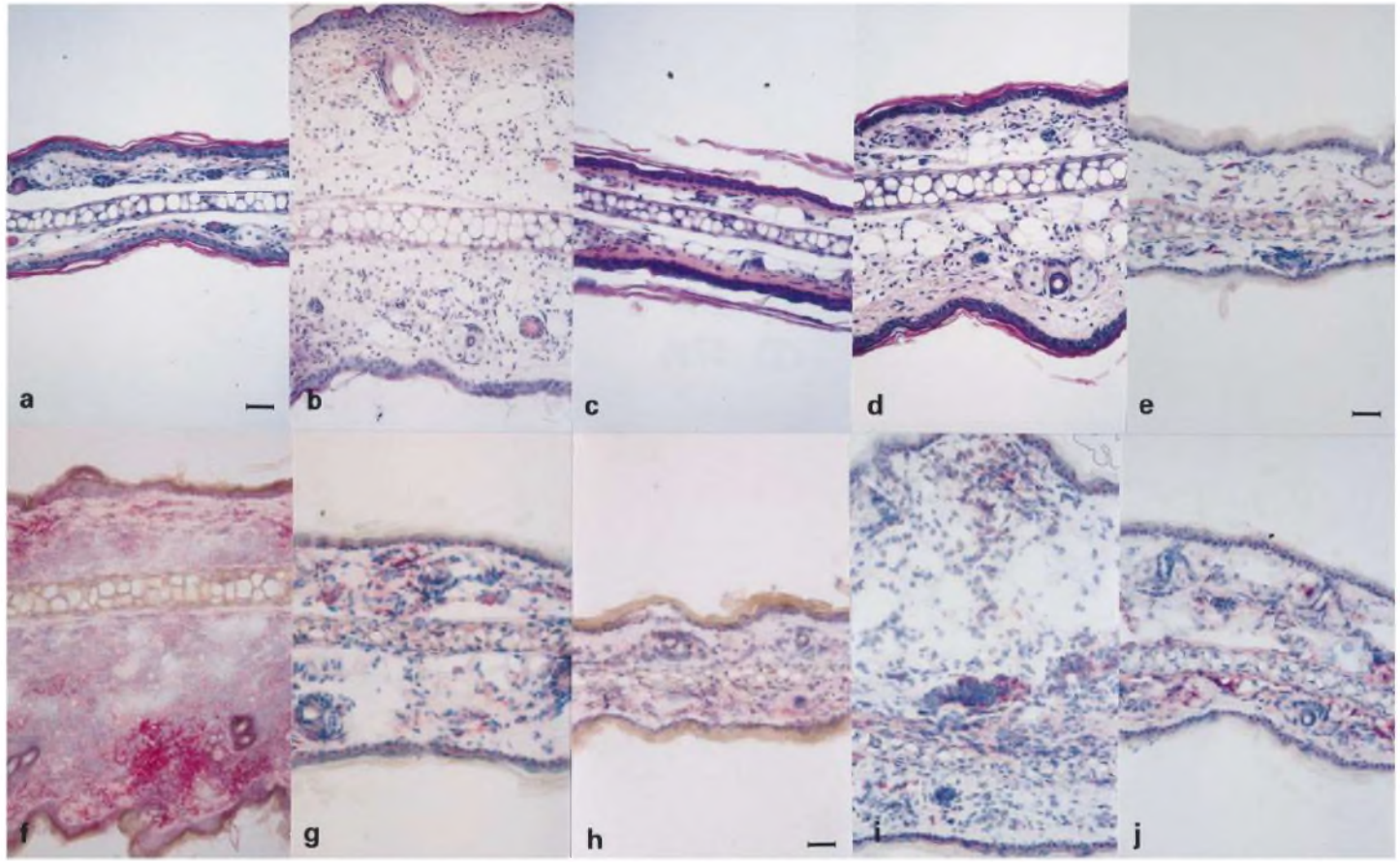

Figure 2. Histologic analysis of the CHS response in IL-4 deficient mice. Cellular infiltrate and edema is reduced in IL-4-deficient mice (*A–D*) and accompanied by decreased HLA class II (*E–G*) and ICAM-1 expression 24 h after challenge (*H–J*). Histologic analysis of the CHS response: (*A*) wild-type ear, unchallenged (*B*), wild-type ear 24 h after challenge with 1% DNCB. Ear thickening is due to edema and infiltration of inflammatory cells, (*C*) IL-4-deficient mice ear, unchallenged, (*D*) IL-4-deficient mice ear, 24 h after challenge with 1% DNCB. A moderate edema and a lower cellular infiltrate as in wild-type mice leads to the reduced ear thickness. Immunohistochemistry for HLA class II: (*E*) wild-type ear unchallenged (mutant ear not shown); (*F*) wild-type ear 24 h after challenge with 1% DNCB; (*G*) IL-4-deficient mice ear 24 h after challenge, note the reduced edema and infiltrate accompanied by decreased staining for HLA class II in the infiltrate. Immunohistochemistry for ICAM-1: (*H*) wild-type ear unchallenged (mutant ear not shown); (*I*) wild-type ear 24 h after challenge with 1% DNCB; (*J*) IL-4-deficient mice ear 24 h after challenge, please note the reduced edema and infiltrate accompanied by decreased staining for ICAM-1. *Scale bar*: 40 μm.

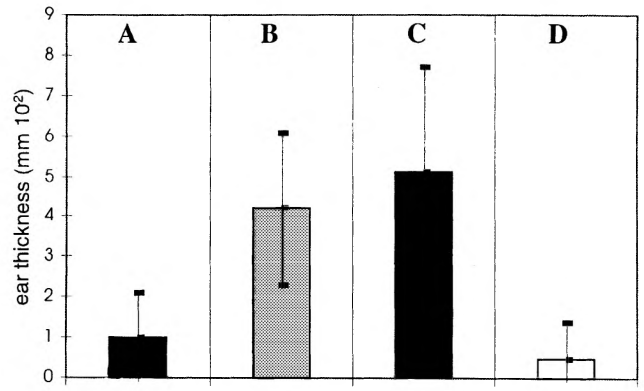

Figure 3. Lymph node cells from interleukin-4 deficient mice fail to induce CHS in BALB/C mice Adoptive transfer experiments were performed: lymph node cells from sensitized mutant mice were injected subcutaneously into the ears of BALB/C mice and vice versa. Mice were challenged immediately after injection of lymph node cells with 1% DNCB. Data represent mean swelling values obtained after 12 h with five mice per group and are representative of two independent experiments (statistical significance: $p < 0.02$). (*A*) Lymph node cells of sensitized IL-4-deficient mice fail to mediate CHS in BALB/C mice. (*B*) Lymph node cells from wild-type mice mediate CHS in IL-4-deficient mice. As a control is shown ear swelling after sensitization of wild-type mice (*C*) and IL-4 mutant mice (*D*).

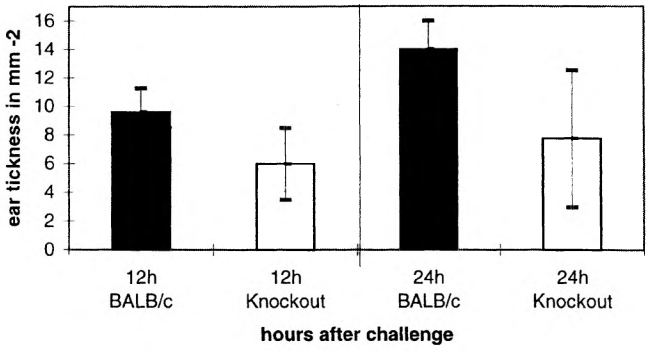

Figure 4. CHS to oxazolone is not reduced in IL-4-deficient mice. Control mice (BALB/C) and IL-4-deficient mice were sensitized with 1% oxazolone on the right ear and vehicle alone on the left ear. Ear swelling was measured after 10 and 24 h. Results are presented as the difference of the ear thickness right and left to each time point. (–/–) IL-4-deficient mice; (+/+): control mice.

IL-4-deficient $CD8^+$ T cells produced significantly more IL-10 than cells obtained from control mice (**Fig 6*a***).

DISCUSSION

The duration and magnitude of the CHS response depend on cell–cell interactions, which are generally mediated by a direct contact via receptor–ligand interactions and indirect signaling via cytokines or growth factors. Concerning the role of IL-4 in CHS contradicting results have been obtained (Gautam *et al*, 1992; Salerno *et al*, 1995; Asada *et al*, 1997) supporting an anti-inflammatory as well as a proinflammatory function of IL-4. While Gautam *et al* (1992) demonstrated that injection of recombinant IL-4 protein inhibits the elicitation phase and treatment of mice with anti-IL-4 monoclonal antibody augmented the CHS response, Salerno *et al* (1995) observed that the treatment of lymph node cells with anti-sense oligonucleotides to IL-4 inhibited the transfer of CHS to recipient mice.

Our study aimed to analyze the role of endogenous IL-4 in CHS using transgeneic mice lacking IL-4 expression. Therefore, we induced CHS in BALB/C mice bearing a germline mutation in the IL-4 gene using two different obligate contact allergens:

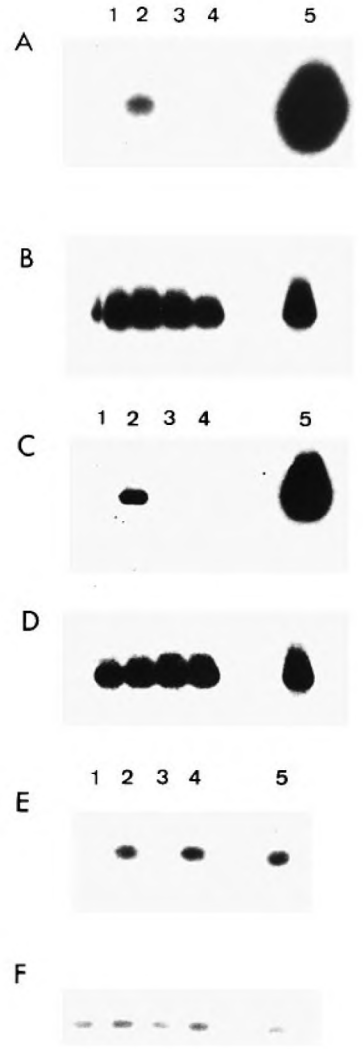

Figure 5. Lack of IL-10 mRNA induction in the CHS response of IL-4-deficient mice. Mice sensitized with 5% DNCB were challenged with 1% DNCB on the right ear and vehicle alone on the left ear. *Lane 1*, wild-type mice control (i.e., left ear exposed to vehicle alone); *lane 2*, wild-type mice challenge (i.e., right ear challenged with 1% DNCB (*A–D*) or oxazolone (*E, F*); *lane 3*, IL-4-deficient mice control (i.e., left ear exposed to vehicle alone); *lane 4*, IL-4-deficient mice challenge (i.e., right ear challenged with 1% DNCB (*A–D*) or oxazolone (*E, F*); *lane 5*, positive control consisting of RNA extracted from Hy 358 cells transfected with the murine IL-4 gene (*A, B*) and IL-10 gene (*C–F*). (*A*) IL-4 mRNA expression was assessed 24 h after challenge confirming a strong induction of IL-4 mRNA expression in wild-type mice (*lane 2*), and no expression in IL-4-deficient mice (*lane 4*). (*B*) GAPDH mRNA expression is shown as a control. (*C*) IL-10 mRNA expression was determined after 12 h showing a strong induction in the control mice (*lane 2*), whereas this induction is lacking in the IL-4 deficient mice (*lane 4*). (*D*) GAPDH mRNA expression is shown as a control. (*E*) IL-10 mRNA expression is both induced in control mice (*lane 2*) and mutant mice (*lane 4*) after challenge with 1% oxazolone. (*F*) GAPDH mRNA expression is shown as a control. The blots are representative of three independent experiments. PCR for each cytokine was carried out at least three times

DNCB and oxazolone. CHS was measured quantitatively by using the classic parameter of contact dermatitis, the ear swelling test (Gad *et al*, 1986). Previously, Berg *et al* (1995) reported a nonsignificant reduction of the CHS to oxazolone in IL-4-deficient mice on the C57BL/6 background, which is known to be a low responder in CHS (Shultz and Baily, 1975). Weigmann *et al* (1997) observed a significant reduction of CHS in the late phase (up to day 3 after challenge) of the CHS response using DNFB as the allergen in the

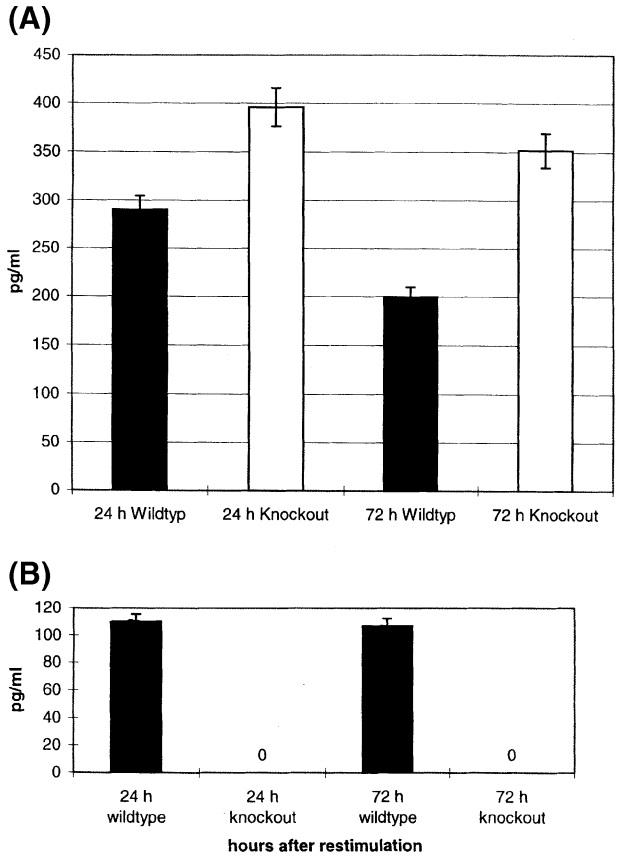

Figure 6. Differential induction of cytokine synthesis after hapten sensitization. (*A*) Higher concentrations of IL-10 measured in supernatants of $CD8^+$ T cell lines from IL-4-deficient mice when compared with control mice ($p < 0.02$). (*B*) Concentration of IL-4 in supernatants of DNBS-specific $CD4^+$ cell lines from IL-4-deficient and wild-type mice: maximal production of IL-4 of $CD4^+$ IL-4 (+/+) T cells 1 d after restimulation with DNBS modified spleen cells. No detectable IL-4 in supernatants of T cell lines from deficient mice. Data represent mean concentration values obtained from supernatants from five wells each and are representative of two independent experiments.

same strain (C57BL/6). BALB/C mice, however, which have a different genetic restriction and are widely used to investigate CHS reactions, were not yet analyzed. This mouse strain is known as a high responder in contact dermatitis (Shultz *et al*, 1975).

Using DNCB as the sensitizing allergen, the time course of CHS in BALB/C mutant mice and control mice was characteristic for a CHS response with maximum ear swelling after 24 h. This indicates that contact dermatitis can be induced in mutant BALB/C mice lacking IL-4 expression. Direct comparison of the intensity of the ear swelling response of mutant and control mice, however, revealed that mutant mice showed a significantly attenuated and shortened CHS response from 12 to 194 h after challenge. Histologic examination of the ears revealed that the impaired CHS response in IL-4-deficient mice measured by the mouse ear swelling test was accompanied by decreased cellular infiltrate and edema in the challenged ear. The reduced cellular infiltrate was also accompanied by a diminished induction of ICAM-1 and major histocompatibility complex class II molecules, which could in part be due to the lacking action of IL-4, as this cytokine has previously been shown to induce these molecules (Masinovsky *et al*, 1990; Galea *et al*, 1993).

The attenuated CHS response in the IL-4-deficient mice was restored to normal levels in heterozygous mice excluding an influence of the genetic background. These findings do not support an inhibitory role of IL-4 in the CHS response to DNCB but rather a proinflammatory, as both magnitude and duration of the CHS response were significantly diminished in the IL-4-deficient mice. As our results differ from the observations of Berg *et al* (1995) the question arose whether the allergens (oxazolone, DNCB) applied or the genetic background is responsible for this discrepancy. Therefore, additional experiments were performed using oxazolone as the sensitizing agent. Notably, as in the C57BL/6 strain, no difference in the ear swelling response compared with wild-type mice was observed. Thus, our results extend the observations of Berg *et al* (1995) suggesting that the CHS response to oxazolone and DNCB evolves on different pathways independent and dependent on IL-4. Similar results have recently been obtained by Shornick *et al* (1996) and Zengh *et al* (1995). These authors reported that IL-1β-deficient mice exhibited a differential response to trinitrochlorobenzene (TNCB) and oxazolone. A normal CHS response to oxazolone was found, whereas a defective CHS response to low doses of TNCB could be restored using high doses of the sensitizing antigen. Therefore, one may speculate that more than one type of CHS exists or rather different agents induce different immunologic pathways and cytokine cascades. This has already been implicated for irritants (Willis *et al*, 1993; Willmer *et al*, 1994; Grangsjo *et al*, 1996).

To investigate whether an impaired antigen-specific proliferation is responsible for the attenuated CHS in IL-4-deficient mice proliferation assays of $CD4^+$ and $CD8^+$ T cells were performed. These experiments revealed that antigen-specific proliferation of $CD4^+$ and $CD8^+$ T cells is unaltered in the absence of endogenous IL-4. Weigmann *et al* (1997) showed that the antigen presentation of Langerhans cells in IL-4-deficient mice is unaltered suggesting that neither an impaired proliferation capacity of T cells nor an impaired antigen presentation from Langerhans cells to T cells is responsible for the reduction of CHS in IL-4-deficient mice. The analysis of the supernatants of antigen-specific $CD4^+$ and $CD8^+$ T cells from IL-4-deficient and control mice revealed a differential pattern of cytokine synthesis. Although IL-2 and IL-1β production was similar in IL-4 +/+ and IL-4 −/− mice, supernatants from $CD8^+$ cells in IL-4-deficient mice showed higher IL-10 concentrations, whereas in mice on a different genetic background (Kopf *et al*, 1993) a deficiency of IL-10 synthesis had been observed. Interestingly, adoptive transfer experiments revealed that no CHS response could be elicited when lymph node cells from sensitized IL-4-deficient mice were injected into the ears of control mice. This indicates that IL-4 is an important constituent of the cytokine milieu during the early effector phase, as it has been previously suggested by the studies of Salerno *et al* (1995). Further studies are needed, however, e.g., by reconstituting IL-4 in the mutant mice, to examine its exact role further.

The reduced ear swelling and inflammatory response after challenge with 1% DNCB is accompanied by a lack of IL-10 mRNA expression in IL-4-deficient mice. This indicates that overproduction of IL-10 in the ear skin, which is thought to be predominantly produced by keratinocytes (Ferguson *et al*, 1994), is not responsible for the diminished CHS response. The lacking induction of IL-10 in IL-4-deficient mice rather reflects the largely reduced inflammatory reaction after challenge with DNCB, as IL-10 is normally induced after challenge with oxazolone. Although an induction of other inhibitory cytokines cannot be excluded, our results further provide strong evidence for a direct proinflammatory activity of IL-4 during the early phase of CHS response as proposed by other authors (Müller *et al*, 1993; Trepper, 1994; Salerno *et al*, 1995).

In summary this study shows that, unexpectedly, a loss of endogenously produced IL-4 leads to a significant reduction of the magnitude and duration of the CHS response to DNCB supporting an important proinflammatory function of this cytokine. The normal CHS response to oxazolone in IL-4-deficient mice suggests, however, that this proinflammatory role depends on the chemical compound used to elicit the CHS response.

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REFERENCES

Asada H, Linton J, Katz SI: Cytokine gene expression during the elicitation phase of contact sensitivity: regulation by endogenous IL-4. *J Invest Dermatol* 108:406–411, 1997

Berg DJ, Leach MW, Kühn R, *et al*: Interleukin-10 but not interleukin-4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* 182:99–108, 1995

Cher DJ, Mosmann TR: Two types of murine helper T-cell clone. II. Delayed type hypersensitivity is by mediated Th-1 clones. *J Immunol* 138:3688, 1987

Dieli F, Asherson GL, Romano GC, Sireci G, Gervasi F, Salerno A: IL-4 is essential for the systemic transfer of delayed hypersensitivity by T-cell lines. *J Immunol* 152:2698–2704, 1994

Enk AH, Angeloni VL, Udey MC, Katz SC: Inhibition of DC function by IL-10: a role for the induction of tolerance. *J Immunol* 151:2390–2396, 1993

Erb KJ, Rüger B, Brevern M, Ryffel B, Schimple A, Rivett K: Constitutive expression of interleukin-4 in vivo causes autoimmune type disorders in mice. *J Exp Med* 185:329–330, 1997

Fanslow WC, Clifford KN, Park LS, Rubin AS, Voice RF, Beckmann MP, Widner MB: Regulation of alloreactivity in vivo by IL-4 and the soluble IL-4 receptor. *J Immunol* 147:535–542, 1991

Ferguson TA, Dube P, Griffith TS: Regulation of contact hypersensitivity by interleukin-10. *J Exp Med* 179:1597–1604, 1994

Gad SC, Dunn BJ, Dobbs DW, Reily C, Walsh RD: Development and validation of an alternative dermal sensitization test: the mouse ear swelling test. *Toxicol Appl Pharmacol* 84:93–114, 1986

Galea P, Lebranchu Y, Thibault C, Baros P: Interleukin-4 and TNF alpha induce differential adhesion pathways of endothelial cells for the binding of peripheral blood lymphocytes. *Scand J Immunol* 36:575, 1993

Gautam S, Matraiano N, Chikkala M, Edinger M, Tubbs R: L3T4 ($CD4^+$) T-cells that mediate contact hypersensitivity to trinitrochlorobenzene express I-A determinants. *Cell Immunol* 135:27–41, 1991

Gautam S, Chikkala M, Hamilton TA: Anti-inflammatory action of IL-4: Negative regulation of contact sensitivity to TNCB. *J Immunol* 148:1411–1415, 1992

Goldman M, Druet D, Gleichmann E: Th2 cells in systemic autoimmunity: insights from allogeneic diseases and chemically induced autoimmunity. *Immunol Today* 12:223–228, 1991

Gosinski BL, Tigelaar RE: Roles of CD4+ and CD8+ T-cells in murine contact hypersensitivity revealed by in vivo monoclonal antibody depletion. *J Immunol* 144:4121–4128, 1990

Grangsjo A, Leijon-Kuligowski A, Torma H, Roomans GM, Lindberg M: Different pathways in irritant contact excema? Early differences in the epidermal elemental content and expression of cytokines after application of 2 different irritants. *Contact Dermatitis* 35:355–360, 1996

Hauser C: Cultured epidermal Langerhans cells activate effector T-cells for contact sensitivity. *J Invest Dermatol* 95:436–440, 1990

Kopf M, Le Gros G, Bachmann M, Lamers M, Bluethmann H, Köhler G: Disruption of the murine IL-4 gene blocks Th2 cytokine response. *Nature* 362:245–256, 1993

Kühn R, Rajewsky K, Müller W: Generation and analysis of interleukin-4 deficient mice. *Science* 254:707–710, 1991

Masinovsky B, Urdal D, Gallatin M: IL-4 acts synergistically with IL-1 to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *J Immunol* 145:2886, 1990

Miller SD, Jenkins MK: In vivo effects of GK1.5 (anti-L3T4a) monoclonal antibody on induction and expression of delayed hypersensitivity. *Cell Immunol* 92:414–426, 1985

Mosmann TR, Coffmann RL: Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol* 46:111–139, 1989

Mueller R, Krahl T, Sarvetnick N: Tissue specific expression of interleukin-4 induces extracellular matrix accumulation and extravasation of B-cells. *Lab Invest* 76:117–128, 1997

Müller KM, Jaunin F, Masouye I, Saurat JH, Hauser C: Th2 cells mediate IL-4 dependent local tissue inflammation. *J Immunol* 150:5576–5584, 1993

Salerno A, Dieli F, Sireci G, Bella via A, Asherson GL: Interleukin-4 is a critical cytokine in contact hypersensitivity. *Immunology* 84:404–409, 1995

Shearer GM: Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur J Immunol* 4:527, 1974

Shornick LP, Togni P, Mariathasan S, *et al*: Mice deficient in IL-1 (manifest impaired contact hypersensitivity to Trinitrochlorobenzene. *J Exp Med* 183:1427–1436, 1996

Shultz LD, Baily DW: Genetic control of contact sensitivity in mice: effect of H-2 and non H-2 loci. *Immunogenetics* 1:570–583, 1975

Trepper R: The anti-tumor and proinflammatory actions of IL-4. *Res Immunol* 144:633–636, 1994

Van der Heijden FL, Wierenga EA, Bos JD, Kapsenberg ML: High frequency of IL-4 producing $CD4^+$ allergen specific T lymphocytes in atopic dermatitis lesional skin. *J Invest Dermatol* 97:389–393, 1991

Weigmann B, Schwing H, Huber H, Ross R, Mossmann H, Knop J, Reske-Kunz AB: Diminished contact hypersensitivity response in IL-4 deficient mice at a late phase of the elicitation reaction. *Scand J Immunol* 45:308–314, 1997

Willis CM, Stephens CJM, Wilkinson JD: Differential patterns of epidermal leukocyte infiltration in patch test reactions to structurally unrelated chemical irritants. *J Invest Dermatol* 101:364–370, 1993

Willmer JL, Burleson FG, Kayama F, Kanno J, Luster MI: Cytokine induction in human epidermal keratinocytes exposed to contact irritants and its relation to chemical induced inflammation in mouse skin. *J Invest Dermatol* 102:915–922, 1994

Xu H, Dilulio NA, Fairchild RL: T cell populations primed by hapten sensitisation in contact sensitivity are distinguished by polarised patterns of cytokine production: Interferon (producing (Tc1) effector $CD8^+$ cells and interleukin-4/IL-10 producing (Th2) negative regulatory $CD4^+$cells. *J Exp Med* 183:1001–1012, 1996

Zengh H, Fletcher D, Kozak W, *et al*: Resistance of fever induction and impaired acute phase response in interleukin-1 beta deficient mice. *Immunity* 3:9–19, 1995