IL-12 Breaks Dinitrothiocyanobenzene (DNTB)-Mediated Tolerance and Converts the Tolerogen DNTB into an Immunogen

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*J Immunol* 2005; 175:5866-5874; doi: 10.4049/jimmunol.175.9.5866
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IL-12 Breaks Dinitrothiocyanobenzene (DNTB)-Mediated Tolerance and Converts the Tolerogen DNTB into an Immunogen

Helge Riemann,²* Karin Loser,²* Stefan Beissert,† Mayumi Fujita,* Agatha Schwarz,‡ Thomas Schwarz,§ and Stephan Grabbe³†§

Epicutaneous application of dinitrothiocyanobenzene (DNTB) induces tolerance against its related compound dinitrofluorobenzene (DNFB), because DNTB-pretreated mice cannot be sensitized against the potent hapten DNFB. This tolerance is hapten-specific and transferable. In this study, we demonstrate that IL-12 can break DNTB-mediated tolerance. Furthermore, naive mice treated with IL-12 before DNTB application responded to DNFB challenge with a pronounced ear swelling response without previous sensitization to DNFB, showing that IL-12 can convert the tolerogen DNTB into an immunogen. No differences in numbers or regulatory activity were observed between CD4⁺CD25⁺ regulatory T cells isolated from mice treated with DNFB, DNTB, or IL-12 followed by DNTB. However, the number of CD207⁺ Langerhans cells in regional lymph nodes of DNTB-treated mice was significantly lower than in animals treated with DNFB or IL-12 plus DNTB. Additionally, CD11c⁺ dendritic cells (DC) isolated from regional lymph nodes of DNTB-treated mice had a significantly lower ability to stimulate T cell proliferation and produced reduced amounts of inflammatory cytokines. Application of both DNFB and DNTB induced apoptotic cell death of DC in the epidermis and the regional lymph nodes. However, the number of apoptotic DC in regional lymph nodes was significantly higher in DNTB-treated animals compared with mice treated with DNFB or IL-12 plus DNTB. Therefore, we conclude that DNTB-mediated tolerance is secondary to inefficient Ag presentation as a result of apoptotic cell death of DC and that IL-12 converts the tolerogen DNTB into an immunogen by preventing DNTB-induced apoptosis of DC. The Journal of Immunology, 2005, 175: 5866–5874.

The heterodimeric cytokine IL-12 is composed of covalently linked 35- and 40-kDa subunits (1). Besides stimulatory effects on both, NK cells and CD8⁺ cytotoxic T lymphocytes, IL-12 has costimulatory and regulatory effects on CD4⁺ Th cells and favors the differentiation of Th1 cells (2–4). Although contact hypersensitivity (CHS) is unaltered in IL-12-deficient mice, exogenous IL-12 can enhance the CHS response by amplifying the development of hapten-specific CD8⁺ T cells and by inhibiting the induction of Ag-specific CD4⁺ regulatory cells (5, 6). Furthermore, neutralization of IL-12 by injection of blocking Abs inhibits the induction of CHS in wild-type mice (7) and even induces hapten-specific tolerance (8). In contrast, administration of exogenous IL-12 can overcome UV-induced immune tolerance (9, 10). Epicutaneous application of haptens onto mice that have been exposed to UV radiation does not result in sensitization but induces tolerance (11). This tolerance is hapten-specific and can be adoptively transferred by injecting bulk T cells obtained from animals tolerized in this manner (12). We and others have reported that IL-12 is able to prevent UV-induced immunosuppression when injected i.p. into mice between UV exposure and hapten sensitization (7, 9, 10). In addition, tolerance did not develop in these animals (9). Even more importantly, mice tolerized by hapten application on UV-irradiated skin can be fully sensitized with the same hapten when IL-12 is injected before re-sensitization, demonstrating that IL-12 cannot only prevent but also break established UV-mediated tolerance (9, 10). Although IL-12 seems to act on regulatory T cells (13), the detailed mechanism by which IL-12 breaks established tolerance remains to be determined.

Epicutaneous application of dinitrothiocyanobenzene (DNTB) has been reported to induce tolerance against its related compound dinitrofluorobenzene (DNFB) because DNTB-pretreated mice cannot be sensitized against the potent hapten DNFB (14, 15). Specific immune tolerance is induced by topical application of DNFB 7 days before sensitization to DNFB. Tolerance can be abrogated by cyclophosphamide, indicating that DNTB-induced suppressor/regulatory T cells may be involved, and adoptive transfer studies showed that DNTB induces hapten-specific regulatory T cells (15). In addition, lymph node cells from DNTB-treated mice are defective in proliferation and produce significantly lower amounts of IL-1, IL-2, and IL-4 compared with DNFB-treated animals, suggesting that DNTB-induced tolerance results in deficient Th1 as well as deficient Th2 responses to hapten re-exposure (16).

Here, we show that injection of IL-12 into DNTB-treated mice before sensitization with DNFB restores CHS responses and that injection of IL-12 before DNTB treatment enables subsequent sensitization against DNFB. In addition, naive mice are injected

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Received for publication January 1, 2005. Accepted for publication August 18, 2005.

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1. This work was supported by grants from the German Research Foundation [DFG Schw 1177/1-1 (to A.S.), SFB 415/6-A16 (to T.S.), SFB 293-B1 and SFB492-B2 (to S.G.), DFG 1580/6-2 and SFB 293-B8 (to S.B.), and IZKF Lo2/065/04 (to K.L. and S.B.)].
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4. Abbreviations used in this paper: CHS, contact hypersensitivity; CBA, cytometric bead array; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; DNTB, 2,4-dinitrothiocyanobenzene; LC, Langerhans cell.

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0022-1767/05/$02.00
with IL-12 followed by DNTB treatment, primary contact with DNFB results in a specific ear swelling response, indicating that IL-12 converts the tolerogen DNTB into an immunogen. Furthermore, we show that treatment with DNTB leads to increased numbers of apoptotic dendritic cells (DC) both in epidermis and regional lymph nodes and that this effect can be mitigated by IL-12.

Materials and Methods

Mice

BALB/c mice between 8 and 12 wk of age were purchased from Charles River Laboratories. Animals were housed under specific pathogen-free conditions and treated according to institutional guidelines.

Contact hypersensitivity

Mice were sensitized by painting 25 μl of 2,4-dinitrofluorobenzene solution (Sigma-Aldrich; 0.5% in acetone/olive oil 4:1) on the shaved back on day 0. On day 5, the left ear was challenged by applying 20 μl of 0.3% DNFB, and the right ear was treated with acetone/olive oil alone. Ear swelling was measured in a blinded fashion with a spring-loaded micrometer (Mitutoyo) 24 h after challenge. Contact hypersensitivity was determined as the amount of swelling of the hapten-challenged ear compared with the thickness of the vehicle-treated ear and was expressed in centimeters × 10^-3 (mean ± SD). Mice that were ear challenged without previous sensitization served as negative controls. Sensitization against oxazolone was performed by applying 100 μl of a 2% oxazolone solution on day 0, and ears were challenged using 20 μl of 0.5% oxazolone on day 5. Each group consisted of at least 5 mice, experiments were performed at least three times.

DNTB treatment

Mice were painted with 100 μl of DNTB (Lancaster Synthesis) solution (1% in acetone/olive oil 4:1) on the shaved abdomen. On day 7, mice were sensitized with 0.5% DNFB, and DNFB challenge was performed 5 days later.

Injection of rIL-12

For in vivo injection, 250 ng of recombinant murine IL-12 (Genetics Institute) diluted in sterile endotoxin-free saline (PAA) were used. The cytokine was injected i.p. 24 and 3 h before treatment with DNTB or DNFB. Control mice were treated i.p. with equal volumes of saline, which had no effect on the outcome of the sensitization procedure or on the suppressive effect of DNTB.

Adoptive transfer of immune response

Donor mice were treated with DNTB, draining lymph nodes and spleens were removed 7 days later, and single cell suspensions were prepared. Cell number was adjusted to 5 × 10^6 cells/ml, and 200 μl (1 × 10^6 cells) were injected i.v. into naive recipients. Recipients were sensitized 24 h after injection by painting 0.5% DNFB on the shaved back. Recipient mice were challenged on the left ear 5 days later, and ear swelling was evaluated.

Immunofluorescence and in situ TUNEL analysis

Immunofluorescence stainings were performed on cryostat sections of mouse ears according to standard methods (17). Epidermal Langerhans cells (LC) were identified using anti-CD207 (clone 929F3, diluted 1/50 in PBS; kindly provided by Dr. S. Saeland, Schering-Plough, Dardilly, France) and OregonGreen-coupled secondary Ab (Molecular Probes). Apoptosis of Langerhans cells was detected using TUNEL staining and the Texas Red in situ cell death detection kit (Roche) according to the manufacturer’s instructions. Slides were examined using an Olympus BX61 microscope and the MetaMorph software (Visitron Systems).

Cell preparation and flow cytometry

Single cell suspensions of regional lymph nodes were prepared as described before (18). DC were isolated from lymph node cell suspensions by MACS (Miltenyi Biotec) using anti-CD11c-coupled microbeads according to the manufacturer’s instructions. Expression of cell surface and intracellular markers was analyzed by standard four-color flow cytometry with a FACS Calibur flow cytometer and CellQuest software (BD Pharmingen). Cells were stained for FACS analysis in PBS containing 1% FCS with the following mAbs: anti-neuropilin-1 (clone H-286; Santa Cruz Biotechnology), FITC-conjugated anti-CD103 (clone 2E7), anti-CD80 (clone 16-10A1), goat anti-rabbit Ig (Dianova), PE-conjugated anti-CD25 (clone PC61), anti-CTLA-4 (clone UC10-4F10-11), anti-CD86 (clone GL1), peridinin chlorophyll protein-conjugated anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5), allophycocyanin-conjugated anti-CD4 (clone RM4-5), anti-CD25 (clone PC61), anti-CD11c (clone HL3), Cy5-conjugated anti-CD207 (Langerin, clone 929F3; kindly provided by Dr. S. Saeland, Schering-Plough, Dardilly, France). CD207 staining was performed after cell permeabilization. Isotype-controlled match Abs were included in each staining. All Abs as well as isotype-matched controls were obtained from BD Pharmingen unless otherwise noted. Apoptotic and necrotic cells were identified using the Annexin V apoptosis detection kit (BD Pharmingen) according to the manufacturer’s instructions.

Proliferation assays

Naive CD4+CD25- and CD4+CD25+ cells were sorted by MACS as described (18). Proliferation assays were performed in triplicate, and T cell proliferation was assessed by [3H]thymidine incorporation. CD4+CD25- and CD4+CD25+ T cells (2 × 10^6/ml alone or mixed at indicated ratios) were cultured in 96-well round-bottom plates, and cells were stimulated with 1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51). Proliferation assays were done for 96 h in a final volume of 200 μl; 1 μCi/well [3H]thymidine was added for the last 12 h of the experiment, and thymidine incorporation was measured by liquid scintillation counting.

Mixed lymphocyte reactions

Proliferation of CD4+CD25- T cells isolated from lymph nodes of naive BALB/c mice was assessed by [3H]thymidine incorporation. Cells (1 × 10^6/ml) were cultured in triplicate in 96-well round-bottom plates in the presence of 1 × 10^6 DC isolated from lymph nodes of naive BALB/c mice or mice treated with DNFB, DNTB, or IL-12 followed by DNTB. Mixed lymphocyte reactions were done for 72 h in a final volume of 200 μl. T cell proliferation was evaluated by adding 1 μCi/well [3H]thymidine for the last 12 h of the experiment, and thymidine incorporation was measured by liquid scintillation counting.

Cytometric bead array (CBA)

The cytokine activity in culture supernatants of CD11c+ cells from skin draining lymph nodes of DNFB-sensitized, DNTB-sensitized, and IL-12-treated, and DNTB-sensitized mice was assayed by CBA (BD Pharmingen) according to the manufacturer’s instructions. Cells (2 × 10^6/ml) were incubated for 3 days without any further stimulation at 37°C and 5% CO2 in 96-well round-bottom plates (BD Falcon) in a volume of 200 μl of RPMI 1640 containing 10% FCS. Supernatants were collected and subjected to cytokine quantification using CBA kits.

Statistical analysis

Data were analyzed by Student’s t test and differences were considered significant at p < 0.05. Each experiment was performed at least three times.

Results

Induction of hapten-specific tolerance against DNFB by epicutaneous application of DNTB

DNTB (1%) was painted on the shaved abdomen of BALB/c mice. A sensitizing dose of DNFB was applied 7 days later epicutaneously on the shaved back and challenge with DNFB was performed 5 days thereafter. Although animals sensitized with DNFB showed a significant ear swelling response upon challenge, animals treated with DNTB could not be sensitized with DNFB, as indicated by the absent ear swelling response. Likewise, animals challenged with DNFB without prior sensitization showed no ear swelling response (Fig. 1A). The tolerizing effect of DNTB appeared to be dose dependent, because both lower and higher concentrations of DNTB failed to induce tolerance (data not shown).

DNTB-mediated tolerance against DNFB is transferable

Next we analyzed whether DNTB-mediated tolerance was transferable. Therefore, 1% DNTB was painted on the shaved abdomen of BALB/c mice. Animals were sacrificed 7 days later, and regional lymph nodes and spleens were removed. Single cell suspensions were prepared as described and injected i.v. into naive recipient BALB/c. Recipients were sensitized with DNFB 1 day...
conversion of a tolerogen into an immunogen by IL-12

A DNTB Sensi DNB Chal DNB
- + +
- - +
+ + +

Increase in Ear Thickness (cm \times 10^{-3})

B Donor Recipient
DNTB Sensi DNB Chal DNB
- + +
+ + +
- - +

Increase in Ear Thickness (cm \times 10^{-3})

C IL-12 DNTB Sensi Oxa Chal Oxa
- - +
- - -
- + +
+ - +
+ + -

Increase in Ear Thickness (cm \times 10^{-3})

FIGURE 1. A, DNTB induces tolerance against DNB. Mice were tolerized by epicutaneous application of 100 μl of 1% DNTB on the abdomen, followed by sensitization against DNB (25 μl of 0.5% painted on the back) 7 days later. Ears were challenged with DNB (20 μl 0.3%) 5 days after sensitization with DNB. B, DNTB-mediated tolerance is transferable. Donor mice were painted with DNTB or left untreated, and single cell suspensions were prepared from draining lymph nodes and spleens 7 days later. Cells (1 × 10^6) were injected i.v. into naive recipients followed by DNB sensitization 1 day after transfer. Recipient mice were ear challenged with DNB 5 days after sensitization. C, DNTB-induced tolerance is hapten-specific; IL-12 followed by DNTB does not allow for an unspecified challenge. Mice were treated with DNTB, and a sensitizing dose of oxazolone was applied 7 days later. One group of mice was injected with IL-12 followed by DNTB application but not sensitized against oxazolone. All mice were challenged with oxazolone 5 days after sensitization with oxazolone. Ear swelling response is expressed as the difference (centimeters \times 10^{-3}, mean ± SD) between the thickness of the challenged ear and the vehicle-treated ear. *, p < 0.001 versus positive control; n.s., nonsignificant.

after transfer, and challenge was performed with DNB 5 days after sensitization. Whereas control animals that had received cells from untreated donors showed a significant CHS response upon challenge with DNB, no ear swelling response was observed in recipients of cells from DNTB-treated donors, indicating that these animals could not be sensitized against DNB (Fig. 1B).

DNTB-mediated tolerance is hapten-specific

To exclude that DNTB induces tolerance against an unrelated hapten, mice were treated by epicutaneous application of DNTB onto the shaved abdomen. Sensitization with oxazolone was performed on the shaved back 7 days later, and ear challenge with oxazolone was performed 5 days later. Upon challenge, mice pretreated with DNTB and sensitized with oxazolone had ear swelling responses similar to positive control animals sensitized with oxazolone alone, showing that DNTB-mediated tolerance is specific for DNB (Fig. 1C).

IL-12 prevents DNTB-mediated tolerance

To evaluate whether IL-12 can prevent DNTB-mediated tolerance, IL-12 was injected 24 and 3 h before DNTB application. Seven days later, sensitization with DNB was performed, and ears were challenged with DNB 5 days thereafter. CHS response in DNTB-pretreated mice was significantly suppressed compared with positive control animals. In contrast, when IL-12 was injected 24 and 3 h before DNTB application, the tolerizing effect of DNTB was not observed because animals responded with a marked ear swelling response to sensitization with DNB (Fig. 2A).

IL-12 breaks DNTB-mediated tolerance

To study whether IL-12 can break established DNTB-mediated tolerance, BALB/c mice were treated by epicutaneous application of DNTB. Animals were sensitized with 0.5% DNB 7 days after application of DNTB, and challenge with DNB was performed 5 days later. One group of BALB/c mice received i.p. injections of IL-12, 24 and 3 h before sensitization with DNB. Control animals sensitized with DNB showed a significant ear swelling response upon challenge, whereas animals pretreated with DNTB could not be sensitized against DNB. However, when IL-12 was injected into DNTB-pretreated mice before application of DNB, a significant CHS response was observed (Fig. 2B).

IL-12 converts the tolerogen DNTB into an immunogen

To study whether injection of IL-12 before application of DNTB enables a CHS response to DNB challenge without prior DNB sensitization, mice received IL-12 i.p. 24 and 3 h before DNTB application. Challenge with DNB was performed 7 days later. Although animals treated with DNTB alone showed no ear swelling response upon challenge with DNB, mice treated with the combination of IL-12 injection followed by DNTB application showed a significant CHS response upon challenge with DNB (Fig. 3). This ear swelling response appeared hapten-specific, because the combination of IL-12 injection followed by DNTB application did not allow for a CHS response upon challenge with the unrelated hapten oxazolone (Fig. 1C).

Concomitant application of DNTB and an irrelevant hapten does not reconstitute DNB sensitization

Because DNB sensitizes whereas DNTB induces tolerance against the same hapten, we reasoned that DNTB might fail to generate the immunostimulatory signal(s) necessary for induction of CHS. To substitute for these signal(s), animals were sensitized by epicutaneous application of DNTB in conjunction with the nonrelated hapten oxazolone in the conventional sensitizing dose onto the same area. Ears were challenged with DNB 5 days later. Upon challenge with DNB, no CHS response was observed in
animals sensitized with DNTB alone or in combination with oxazolone (Fig. 4, lines 3 and 4). Thus, unlike IL-12 treatment, concomitant application of an immunologically unrelated hapten could not convert the tolerogen DNTB into an immunogen, suggesting that the tolerogenic effects of DNTB are not due to the lack of an immunostimulatory effect.

**DNTB fails to elicit a CHS response in DNFB-sensitized animals, but CHS reactivity is restored by simultaneous application of an irrelevant hapten**

It has been shown earlier that an inherent feature of contact allergens is their capacity to induce a nonspecific inflammatory response necessary for elicitation of CHS (19). Thus, to investigate whether DNTB, in addition to its tolerogenic effects during hapten sensitization, might also have an altered capacity to elicit CHS responses in DNFB-sensitized mice, animals were DNFB sensitized and subsequently challenged with DNTB. Indeed, application of DNTB also failed to elicit a CHS response in DNFB-sensitized mice, suggesting that it not only lacks sensitizing capacity but also signal(s) necessary for elicitation of a CHS response (Fig. 4, lines 8–10).

We were interested whether concomitant application of a nonrelated hapten could substitute for these signals. Thus, DNFB-sensitized mice were ear challenged with either DNTB alone or with DNTB in combination with a conventional challenging dose of the irrelevant hapten oxazolone. Although DNFB-sensitized animals challenged with DNTB alone failed to develop a CHS response, a significant ear swelling could be observed in animals simultaneously challenged with DNTB plus oxazolone. Moreover, the ear swelling response was clearly dependent on the dose of DNTB applied (Fig. 4, lines 11–13).

**IL-12 does not affect the induction of CD25+CD4+ regulatory T cells**

We next investigated the mechanism by which DNTB might exert its tolerogenic effects during primary hapten exposure. As shown previously, CD4+CD25+ regulatory T cells can control CHS responses (20). To study the role of CD4+CD25+ regulatory T cells in DNTB-induced tolerance, mice were left untreated or were treated with DNFB, DNTB, or IL-12 plus DNTB. Lymph nodes and spleens were removed 7 days later, and single cell suspensions were prepared. CD4+CD25+ and CD4+CD25− cells were separated. CD4+CD25− cells from naive mice were stimulated with anti-CD3 and anti-CD28 Ab in the absence or presence of CD4+CD25+ T cells from animals treated with DNFB, DNTB, or IL-12 plus DNTB. Proliferation was measured by the amount of [3H]thymidine incorporation (Fig. 5A). Whereas CD4+CD25− cells from naive mice alone showed vigorous proliferation, proliferation was significantly inhibited by addition of CD4+CD25+ cells from mice treated with DNFB, DNTB, or IL-12 plus DNTB. DC from DNTB-treated animals fail to prime naive T cells

Mice were treated with DNFB, DNTB, or IL-12 plus DNTB, and DC were isolated from regional lymph nodes. DC were cocultured with CD4+CD25− T cells from naive BALB/c mice for 3
days, and T cell proliferation was measured. Whereas T cells incubated with DC from DNTB-treated animals proliferated vigorously, T cells incubated with DC from DNFB-treated mice showed only a weak proliferative response, suggesting that DNTB fails to activate DC for efficient priming of naive T cells. This effect could partially be reversed by treatment with IL-12 (Fig. 6A).

IL-12 prevents DNTB-induced apoptosis of DC in epidermis and regional lymph nodes

Mice were treated with DNFB, DNTB, or IL-12 plus DNTB, and single cell suspensions were prepared from regional lymph nodes 3 days later. Lymph node cells were stained for CD11c and the Langerhans cell-specific intracellular marker Langerin (CD207) and analyzed by flow cytometry. Although the number of CD11c+ cells was slightly increased, the number of Langerin+ cells was considerably reduced in the skin draining lymph nodes of DNTB-treated compared with DNFB-treated mice (Fig. 6B). Injection of IL-12 into mice before application of DNTB raised the number of treated compared with DNFB-treated mice (Fig. 6C). Interestingly, CD11c+ cells isolated from skin draining lymph nodes of DNTB-sensitized mice were less activated than DC isolated from the lymph nodes of DNFB-sensitized or IL-12 plus DNTB-treated mice as demonstrated by the reduced expression of CD80 and CD86 (Fig. 6D). Moreover, lymph node DC from DNTB-sensitized mice produced reduced amounts of inflammatory cytokines like TNF-α and IFN-γ compared with CD11c+ cells isolated from skin draining lymph nodes of DNFB-sensitized or IL-12 plus DNTB-treated mice (Fig. 6D).

Staining with propidium iodide and annexin V showed that the number of apoptotic CD11c+ cells was increased in lymph nodes from DNTB-sensitized mice compared with control, DNFB, and IL-12 plus DNTB-treated animals (Fig. 7A). To evaluate whether DNTB induces apoptosis of DC not only in regional lymph nodes but also in the skin, mice were treated with DNFB, DNTB, or IL-12 plus DNTB. One day later, skin samples were taken from the area of DNTB, DNFB, or vehicle application. Biopsies were stained for Langerin and for apoptotic cells using the TUNEL assay. Although application of DNFB slightly increased the number of apoptotic cells compared with vehicle-treated skin, the total number of apoptotic cells and especially of apoptotic Langerhans cells was significantly higher after DNTB treatment. However, injection of IL-12 before DNTB application reduced the number of apoptotic cells and especially of apoptotic Langerhans cells significantly higher after DNTB treatment. 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Discussion

DNTB has initially been described to induce tolerance to subsequent treatment with DNFB (14). Because later reports could not confirm the tolerizing capacity of DNTB (21, 22), we first determined whether DNTB in our hands acts as a tolerogen. DNTB at a concentration of 1% applied to the abdomen clearly suppressed subsequent sensitization with DNFB, whereas lower and higher
concentrations failed to exert this effect (data not shown). Therefore, we speculate that the different properties attributed to DNTB are due to different concentrations used and different sites of application (ear vs abdomen). We were able to further consolidate this tolerance model by confirming previous reports that DNTB-mediated tolerance is transferable and that this is due to the development of hapten-specific regulatory/suppressor cells (15).

Once we had established the model of DNTB-mediated tolerance we evaluated the effects of IL-12 at different time points, i.e., after and before tolerance had developed. IL-12 injected after DNTB treatment and just before DNFB application clearly enabled DNFB sensitization, indicating that IL-12 can break DNTB-mediated tolerance. It is important to mention that IL-12 was injected at a time point when regulatory T cells had already developed as demonstrated by adoptive transfer of suppression. These findings are similar to those observed in UV-mediated tolerance (9, 13). We also found that IL-12 is able to prevent DNTB-mediated tolerance, because animals that received IL-12 before DNTB application could be sensitized to DNFB. Furthermore, although animals treated with DNTB alone did not react with ear swelling to DNFB challenge, mice which received IL-12 before DNTB application showed a significant ear swelling response following DNFB application on the ear. Because in these mice, ear challenge with DNFB represents the primary contact with DNFB in a concentration that does not cause ear swelling by itself as shown by the negative controls, the combination of IL-12 plus DNTB obviously sensitizes the animals against DNFB.

Our data suggest that IL-12 specifically prevents immune tolerance, but does not enhance immunity in a general fashion, because injection of IL-12 before DNTB application induced CHS against DNFB, whereas IL-12 in the amounts used throughout this study did not further enhance the induction of CHS if injected before sensitization with DNFB (data not shown). Although this is similar to results we previously obtained in a different mouse strain (9),
IL-12 applied before sensitization did exaggerate CHS responses in other studies, albeit in significantly higher doses (5, 6).

In the model of UV-induced tolerance, the immune suppression seems to be induced by the release of keratinocyte-derived cytokines, especially IL-10 (23). However, the mechanisms by which chemical tolerogens, including DNTB, exert their effects, are poorly understood. Enk and Katz (24) showed that tolerizers in contrast to immunogens do not induce IL-1, MIP-2, IL-10, or class II mRNA. Because we demonstrated that IL-12 can prevent DNTB-mediated tolerance we hypothesized that DNTB might fail to induce IL-12 and thereby induces hapten-specific tolerance. In the human system, induction of IL-12 has been observed upon

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**FIGURE 7.** Detection of apoptotic CD11c+ and Langerhans cells in skin draining lymph nodes and in the skin of sensitized mice. A, Mice were treated with DNFB, DNTB, or IL-12 plus DNTB. Lymph node cells were prepared 3 days later, stained for annexin V and propidium iodide (PI), and subjected to flow cytometry analysis. Cells were gated for CD11c+. Statistical evaluation of apoptotic lymph node DC from five independent experiments is shown. *, p<0.05. B, Immunofluorescence analysis of (i) BALB/c mice as well as (ii) DNFB sensitized, (iii) DNTB-sensitized, or (iv) IL-12 plus DNTB-treated mice. One day after sensitization cryosections of treated skin areas were stained for CD207 (green) and for apoptotic cells (TUNEL assay, red). Apoptotic Langerhans cells are marked with white arrows. Original magnification, ×400. Statistical evaluation of apoptotic Langerhans cells from five independent experiments is shown. In each experiment, numbers of apoptotic LC were determined by two independent investigators in a blinded fashion from 15 randomly selected slide sections. *, p<0.05.
application of haptens (25). However, we were not able to identify either p35 or p40 transcripts in murine skin after hapten application (data not shown). Because IL-12 can prevent DNTB-mediated tolerance, we studied whether DNTB fails to induce IL-12 in the regional lymph nodes and thereby induces tolerance. Both p35 and p40 transcripts could be detected in regional lymph nodes of both DNFB- and DNTB-treated animals (data not shown). Thus, there is little evidence that a failure to induce IL-12 might account for the differences between DNTB and DNFB.

CD4⁺CD25⁺ regulatory T cells provide an important pathway for immune tolerance as shown in different models (20, 26–29). A major reduction in the relative numbers of CD11c⁺ Langerin⁺ DC in regional lymph nodes was observed after application of DNTB when compared with DNFB. Interestingly, injection of IL-12 before DNTB application could raise the number of CD11c⁺ Langerin⁺ cells to levels in the range observed after DNFB treatment. Initially, we speculated that the reduced number of CD11c⁺ Langerin⁺ cells after DNTB treatment could be due to the inability of DNTB to sufficiently activate Langerhans cells for migration to regional lymph nodes. The hapten then would be presented less efficiently, which might lead to tolerance (11, 31). If DNTB failed to activate Langerhans cells to migrate to the lymph nodes, one would expect that simultaneous application of the unrelated hapten oxazolone would substitute for this deficiency as this potent allergen is obviously able to activate DC for migration and efficient Ag presentation. However, concomitant application of oxazolone and DNTB did not lead to induction of CHS. We then analyzed the possibility that DNTB application induces LC damage or even death, thereby decreasing the number of cells that reached the regional lymph node. Indeed, we found that DNTB consistently induced enhanced apoptotic cell death of DC both in epidermis and regional lymph nodes. This toxic, apoptosis-inducing effect of DNTB on APCs is able to explain the dose dependency we observed for the tolerogenic effects of DNTB. Thus, at low concentrations, the damaging effect of DNTB on DC is absent, but the amount of Ag is too small to induce CHS. At high concentrations, apoptotic death of DC will be so extensive that sufficient numbers of viable DC do not reach the regional lymph nodes and thus fail to prime for tolerance. In our study, administration of IL-12 was able to prevent DNTB-induced cell death in vivo. It has recently been shown that IL-12 has anti-apoptotic properties, because it is able to protect keratinocytes from UV-induced apoptosis by inducing DNA repair (32) and to prevent activation-induced cell death in T cells by down-regulating Fas ligand and inhibiting caspase activation (33–35). Now, our findings indicate that IL-12 also protects LC against hapten-mediated apoptosis. Likewise, we demonstrated earlier that UV-mediated tolerance is also dependent on apoptosis of DC and can be reversed by IL-12 (36). Thus, similar mechanisms are responsible for both UV-mediated and hapten-induced tolerance.

In addition to its tolerogenic effects during hapten sensitization, DNTB also failed to elicit a CHS response even in mice that had been sensitized to the relevant hapten by using DNFB. This effect is due to a different mechanism and cannot be explained by induction of LC apoptosis. We have previously shown that for the elicitation of hapten-specific CHS responses, two signals are required: 1) the specific Ag, and 2) a nonspecific proinflammatory signal (19). This conclusion was based on the observation that coadministration of an irrelevant hapten with a low dose of the specific hapten in sensitized mice yielded a full CHS response, whereas application of either substances alone, or the combination of haptens applied to nonsensitized animals, failed to elicit a significant reaction. Although the nature of the proinflammatory signal remains to be characterized, there is strong evidence that this signal is specifically provided by haptens because it could not be substituted by application of irritants (19). To determine whether DNTB differs from hapten in its ability to provide this second signal, mice were either treated by epicutaneous application of DNFB alone or in combination with oxazolone in the induction or effector phase. While in the effector phase a vigorous ear swelling response could be observed in DNFB-sensitized animals challenged with the combination of DNTB and oxazolone, no sensitization against DNFB was observed in animals treated with the combination of DNTB and oxazolone in the induction phase of CHS (Fig. 4). These findings suggest that an absent irritative effect of DNTB could be responsible for the inability of DNTB to elicit a CHS response in DNFB-sensitized animals. In contrast, application of an irrelevant hapten during sensitization did not restore the sensitizing potential of DNTB.

In summary, the present study shows that DNTB acts as a tolerogen upon primary hapten exposure and also fails to elicit CHS responses during secondary hapten application. IL-12 can both prevent and overcome DNTB-mediated tolerance and even convert the tolerogen DNTB into an immunogen. Our data also link the tolerogenic effect of DNTB to induction of apoptosis in LC and demonstrate an anti-apoptotic effect of IL-12 on LC.

Acknowledgments
The excellent technical assistance of Joachim Windau and Maik Voskort is gratefully acknowledged.

Disclosures
The authors have no financial conflict of interest.

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