

# Low zone tolerance induced by systemic application of allergens inhibits T<sub>C</sub>1-mediated skin inflammation

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**Background:** The induction of tolerance may be a promising target of strategies aimed at preventing harmful allergic diseases. Low zone tolerance (LZT), induced by epicutaneous application of low doses of contact allergens, inhibits the development of T<sub>C</sub>1-mediated contact hypersensitivity (CHS). **Objective:** We evaluated the effect of systemic (oral, intravenous) administration of low amounts of haptens on specific immune reactions and tolerance induction. **Methods:** By using the mouse model of LZT, we analyzed immune reactions *in vivo* (skin inflammation) and T-cell responses *in vitro* after oral, intravenous, or epicutaneous application of low amounts of the contact allergen 2,4,6-trinitro-1-chlorobenzene (TNCB). **Results:** Subimmunogenic doses of TNCB applied orally and intravenously induced a significant tolerance reaction *in vivo* comparable to epicutaneously tolerized mice, indicating that LZT is a systemically mediated tolerance reaction. *In vitro* analysis in all models of LZT revealed the generation of IL-10 secreting, regulatory CD4<sup>+</sup> T cells that were absolutely required for the development of hapten-specific CD8<sup>+</sup> T<sub>C</sub>2 cells. Adoptive transfer experiments identified CD8<sup>+</sup> T<sub>C</sub>2 cells as effector T cells of LZT inhibiting the development of CHS-promoting T<sub>C</sub>1 cells and consequently the manifestation of CHS. These suppressor CD8<sup>+</sup> T<sub>C</sub>2 cells were found as well in skin-draining as in mesenteric lymph nodes and in the spleen of tolerized animals independent of the route of tolerization. **Conclusion:** These data indicate that systemic uptake and presentation of small amounts of haptens (eg, contact allergens, drugs, metals) induce the development of LZT and thus prevent inappropriate activation of the immune system and protect from allergic diseases. **Clinical implications:** These findings will be of particular importance because tolerance induction by protocols applying subimmunogenic, low amounts of haptens may be used as tools for immunotherapy in allergic and autoimmune diseases. (J Allergy Clin Immunol 2006;117:1170-7.)

**Key words:** Tolerance, contact allergens, contact hypersensitivity, regulatory T cells, haptens, occupational disease

Among allergic skin diseases, allergic contact dermatitis as the clinical manifestation of contact hypersensitivity (CHS) is one of the major occupational dermatologic problems.<sup>1</sup> The CHS response induced by epicutaneous exposure to contact allergens (haptens) in previously sensitized hosts is mediated by specific CD8<sup>+</sup> T<sub>C</sub>1 cells primed by epidermal Langerhans cells after migration to the regional lymph node.<sup>2-4</sup> Despite considerable progress in elucidating the mechanism of pathogenesis of CHS, efforts to target and develop effective and specific therapeutic strategies are still at initial stages.

Previously, it was shown that low zone tolerance (LZT), induced by epicutaneous application of low, subimmunogenic doses of contact allergens, impedes the development of T<sub>C</sub>1-mediated CHS.<sup>5,6</sup>

Low zone tolerance has been demonstrated to depend largely on the generation of CD8<sup>+</sup> T<sub>C</sub>2 suppressor T cells as effector T cells of LZT that requires the presence of IL-10 secreted by regulatory CD4<sup>+</sup> T cells during the induction of LZT.<sup>7</sup> In contrast with CHS, neither epidermal Langerhans cells nor dermal dendritic cells are mandatory for the induction of LZT.<sup>8</sup>

A long recognized and well established model of acquired peripheral tolerance is the oral tolerance to protein antigens.<sup>9,10</sup> Feeding of high doses of antigen results in T-cell inactivation (anergy) or clonal deletion of antigen specific T cells, whereas application of low doses of protein or peptides induces the development of regulatory T-cell populations, including Tr1 cells, which release large amounts of IL-10, and T<sub>H</sub>3, cells that secrete predominantly TGF- $\beta$ .<sup>11-15</sup>

Haptens (eg, contact allergens, drugs, metals) are low-molecular-weight chemicals that become immunogenic after noncovalent coupling to proteins when entering the organism.<sup>16</sup> It was demonstrated that epicutaneous application of haptens after UV-B radiation or feeding of high doses of contact allergens impedes the development of CHS by specific tolerance reactions that differ from immunologic mechanisms of LZT induced by epicutaneously applied low doses of contact allergens.<sup>17-21</sup>

Because the exposure of organisms to low amounts of allergens represents a naturally occurring process, we evaluated whether LZT represents a systemically induced state of acquired peripheral tolerance. In this study, we demonstrate that, independent of the application route

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#### Abbreviations used

|          |                                     |
|----------|-------------------------------------|
| AOO:     | Acetone/olive oil                   |
| CHS:     | Contact hypersensitivity            |
| ELISPOT: | Enzyme-linked immunosorbent spot    |
| LNC:     | Lymph node cells                    |
| LZT:     | Low zone tolerance                  |
| TNBS:    | 2,4,6-Trinitro-benzenesulfonic acid |
| TNCB:    | 2,4,6-Trinitro-1-chlorobenzene      |
| WT:      | Wild-type                           |

(epicutaneously, orally, and intravenously), low, subimmunogenic doses of contact allergens impede the development of CHS reactions by systemic tolerance induction. In all experimental settings, systemic application of the allergen triggers the development of specific IL-10 secreting regulatory CD4<sup>+</sup> T cells and CD8<sup>+</sup> suppressor T<sub>C</sub>2 T cells. These data provide further evidence for LZT to allergens as an important general mechanism of peripheral tolerance preventing the development of allergic diseases.

## METHODS

### Mice

C57BL/6 wild-type (WT), IL-10<sup>-/-</sup> purchased from Jackson Laboratories (Bar Harbor, Me) and CD4<sup>-/-</sup> mice (strain B6.129S-CD4<sup>tm1Nik</sup>N12) purchased from Taconic Europe (Ry, Denmark) were bred in the animal facility of the University of Mainz.

### Reagents

Picrylchloride (TNCB; 2,4,6-trinitro-1-chlorobenzene) and picrylsulfonic acid (TNBS; 2,4,6-trinitro-benzenesulfonic acid) were purchased from Sigma (Deisenhofen, Germany) and VeZwerf Laborsynthesen (Idar-Oberstein, Germany), respectively.

### Tolerance and CHS induction

*Induction of tolerance (at days 0, 2, 4, 6, and 8).* For epicutaneous tolerance, mice were painted 5 times with tolerizing doses of 4.5 µg TNCB dissolved in 15 µL acetone/olive oil (AOO; vol/vol 3:1) or AOO alone as a control onto shaved areas of the body.

For oral tolerance, experimental groups of mice were fed 5 times with tolerizing doses of 4.5 µg TNCB in 100 µL acetone/PBS (pH 7.0) or acetone/PBS alone as a control using a feeding needle (Acufirm, Dreieich, Germany)

For intravenous tolerance, mice were injected intravenously 5 times into the tail vein with tolerizing doses of 45 µg TNBS dissolved in 100 µL sterile PBS/pH 7.0 or PBS as control.

### Sensitization/effector phase of tolerance

At day 10, all tolerized mice were sensitized by epicutaneous application of 450 µg TNCB in 15 µL AOO and challenged 5 days later by painting 45 µg TNCB onto the dorsal side of the right ear. An increase of ear thickness was measured after 24 hours by using an engineer's micrometer (Oditest; Kroeplin, Schlüchtern, Germany). Results are expressed as mean values in units of millimeters  $\times 10^{-2}$  or percentage of control  $\pm$  SD.

### Preparation of lymph node cells/spleen cells, enrichment of CD8<sup>+</sup> and CD4<sup>+</sup> T cells for adoptive transfer experiments and cell culture

Auricular, cervical, inguinal and, in some experiments as indicated, mesenteric lymph nodes and spleens were taken, and CD8<sup>+</sup> and CD4<sup>+</sup> T cells were positively enriched from whole lymph node cells (LNC) or spleen suspension using CD8-coated or CD4-coated magnetic beads (MACS system; Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany; purity CD8<sup>+</sup>/CD4<sup>+</sup> T cells > 95%). Enriched CD8<sup>+</sup> and CD4<sup>+</sup> T cells were injected intravenously into untreated WT recipients (1 single injection of  $1.5\text{--}2.5 \times 10^7$  CD8<sup>+</sup> or CD4<sup>+</sup> T cells/100 µL PBS). Subsequently, sensitization and challenge were performed as described.

### Hapten modification of LNC

LNC ( $10^7$  cells/mL) were incubated in 10 mmol/L TNBS in HBSS (BioWhittaker, Verviers, Belgium) for 10 minutes at 37°C, subsequently washed 3 times in RPMI-1640, and resuspended in RPMI-1640 supplemented with 2% syngeneic normal mouse serum.

### Cell culture and proliferation assay

Untreated or hapten-modified LNC  $10^6$  per well, resuspended in 200 µL RPMI-1640 supplemented with 2% syngeneic normal mouse serum, were incubated at 37°C/5% CO<sub>2</sub>. After 24 hours of culture, lymph node cells were pulsed for 18 hours with 1 µCi [<sup>3</sup>H] thymidine per well (Amersham Biosciences Europe, Freiburg, Germany). Incorporated radioactivity was determined by using a liquid scintillation counter (1205 Betaplate; LKB Wallac, Bromma, United Kingdom). Results are expressed as mean counts per minute (cpm) of triplicate wells  $\pm$  SDs. For cytokine assessment, T cells ( $5 \times 10^5$ ) or CD8<sup>+</sup> T cells ( $5 \times 10^5$ ) were cocultured with untreated or hapten-modified and irradiated (3000 rad) LNC ( $5 \times 10^5$ ) as antigen-presenting cells.

### Cytokine ELISAs

After 24, 48, and 72 hours, respectively, supernatants of cell cultures were harvested and immediately frozen at  $-80^\circ\text{C}$ . Cytokine ELISAs for IL-2, IL-4, and IL-10 (Becton Dickinson, Heidelberg, Germany) and IFN- $\gamma$  (R&D Systems, Wiesbaden, Germany) were performed according to the manufacturer's instructions.

### Enzyme-linked immunosorbent spot for IL-10 secretion

The enzyme-linked immunosorbent spot (ELISPOT) assay was performed according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, Calif) and as described.<sup>6</sup>

### Statistical analyses

Statistical significances of differences between experimental groups were evaluated by using the Wilcoxon-Mann-Whitney 2-sample test using the Statview 5.0 software package (SAS Institute, Cary, NC). Differences of  $P < .05$  were considered significant.

## RESULTS

### Induction of tolerance to contact allergens after oral or intravenous application of low doses of contact allergens

In this study, we analyzed whether systemic distribution of low doses of allergens induce the development of specific tolerance reaction after epicutaneous as well as after oral or intravenous application. As reported before,

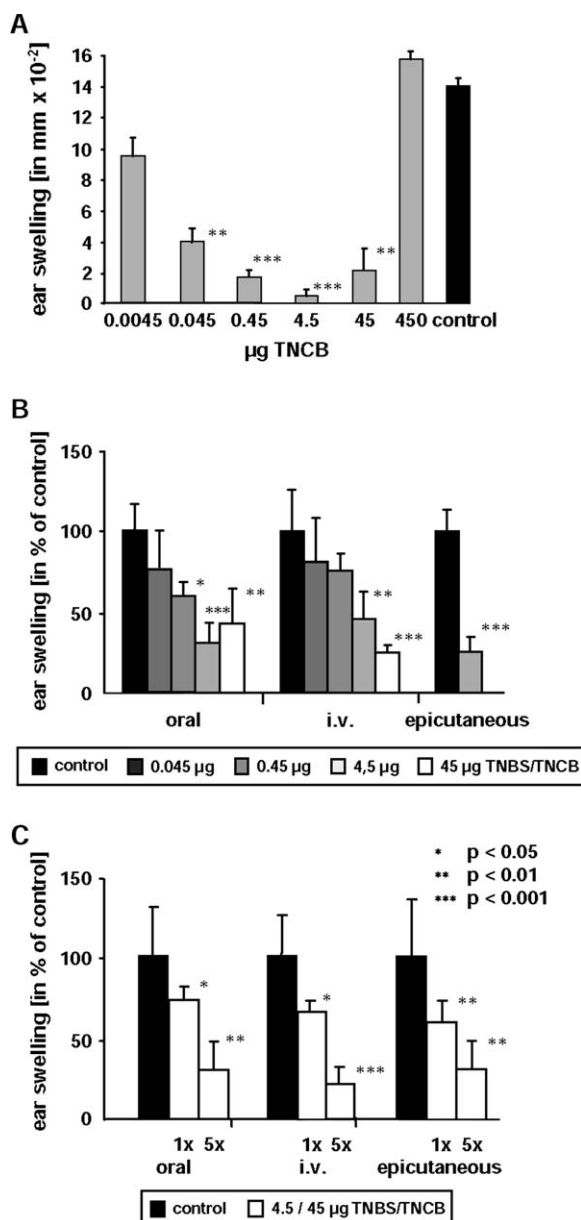


**FIG 1.** Specific tolerance is induced by low doses of orally and intravenously applied allergens. Mice were treated epicutaneously (A) or orally, intravenously or epicutaneously (B, C) with designated doses of TNCB or AOO (control) 5 times (A, B) or as indicated (C). Subsequently, the sensitization protocol was performed as described. Data represent relative changes in ear thickness (means  $\pm$  SDs). Three experiments were performed with similar results.

low subimmunogenic doses of contact allergens induce a state of tolerance when applied epicutaneously, called LZT.<sup>5-8</sup> As described previously and demonstrated in Fig 1, A, these low, subimmunogenic doses of TNCB ( $\leq 45$  µg) applied epicutaneously induced a tolerance reaction as observed by a markedly reduced ear swelling compared with control animals after subsequent sensitization and elicitation of CHS.<sup>5-8</sup>

In the following experiments, we assessed the capacity of low doses of allergens to induce a tolerance reaction

when applied orally or intravenously. On the basis of the results of the epicutaneously induced LZT demonstrated in Fig 1, A, we treated mice with TNCB or TNBS (the water soluble acid of TNCB) in doses ranging from 0.045 µg to 45 µg or PBS (CHS control) by oral (TNCB) or intravenous (TNBS) application. Both oral and intravenous application of these low, subsensitizing doses before sensitization resulted in reduced ear swelling (Fig 1, B). The optimal low allergen dose inducing a significant tolerance reaction was 4.5 µg TNCB by oral and 45 µg TNBS by intravenous application. An enhancement of tolerance reactions was observed after repeated treatments with optimal doses compared with single application of the hapten (Fig 1, C).

### Development and function of regulatory IL-10 secreting CD4<sup>+</sup> T cells in oral and intravenous LZT

During the induction phase of LZT induced by epicutaneously applied contact allergens, IL-10-producing, regulatory CD4<sup>+</sup> T cells are generated as reported previously.<sup>7</sup> To address the questions whether this regulatory CD4<sup>+</sup> T-cell population is also generated after intravenous and oral administration of subimmunogenic doses of haptens, CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations obtained from lymph nodes of tolerized animals were analyzed for IL-10 production by ELISPOT. Our results clearly revealed that after both intravenous and oral tolerization with subimmunogenic doses of allergens, lymph node-derived CD4<sup>+</sup> T cells were the only source of notable amounts of IL-10 in the induction phase of LZT (Fig 2, A). Hapten-specific CD8<sup>+</sup> suppressor T cells and non-T cells do not secrete IL-10 during the induction phase of LZT (Fig 2, A).

To prove formally that these hapten-specific IL-10-secreting CD4<sup>+</sup> T cells display regulatory function and are mandatory for the generation of LZT effector CD8<sup>+</sup> after orally and intravenously induced LZT, we used CD4<sup>-/-</sup> mice and IL-10<sup>-/-</sup> mice for tolerization experiments. Notably, after epicutaneous, oral or intravenous application of low doses of TNCB/TNBS, an unrestricted CHS reaction was observed in CD4<sup>-/-</sup> and IL-10<sup>-/-</sup> mice compared with sensitized control mice, demonstrating the abolishment of tolerance induction (Fig 2, B and C). In addition, no IL-10 release was found in CD4<sup>-/-</sup> mice after tolerization (data not shown). Thus, these results demonstrate a critical regulatory role for IL-10-producing CD4<sup>+</sup> T cells during the induction of LZT to contact allergens (Fig 2, B and C).<sup>7</sup>

### Generation of CD8<sup>+</sup> T<sub>C2</sub> cells during the effector phase of LZT

In addition, we analyzed the T-cell response during the effector phase of LZT. Compared with control sensitized groups, CD8<sup>+</sup> T cells obtained from tolerized mice during the effector phase of LZT exhibited markedly reduced IFN- $\gamma$  release and greatly increased IL-4 and IL-10 secretion after hapten-specific restimulation *in vitro* (Fig 3, A). This T<sub>C2</sub>-skewed cytokine release pattern is characteristic

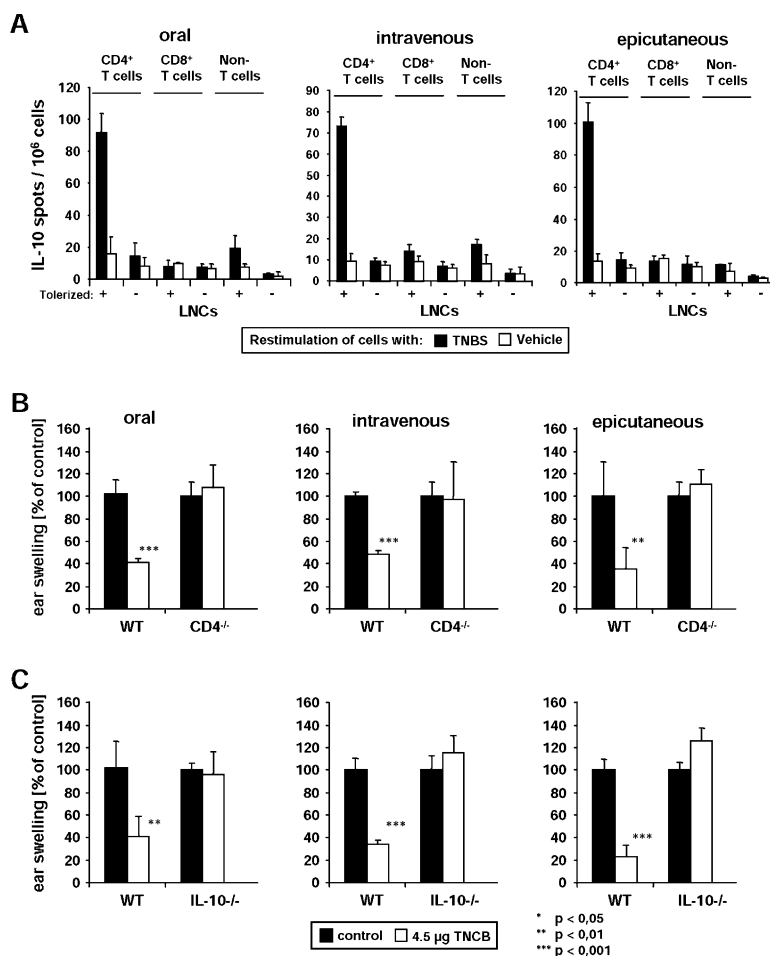


**FIG 2.** IL-10-secreting regulatory CD4<sup>+</sup> T cells are generated during the induction phase of systemically induced LZT. IL-10 release of CD4<sup>+</sup>, CD8<sup>+</sup> T cells, and non-T cells obtained from control or tolerized (epicutaneously, orally, intravenously) mice was assessed by ELISPOT (A). WT and CD4<sup>-/-</sup> (B) and IL-10<sup>-/-</sup> mice (C) were fed, intravenously injected with, or exposed to the indicated doses of TNCB/TNBS. Data represent relative changes in ear thickness (means  $\pm$  SDs).

for impaired CHS responses in the effector phase of LZT as demonstrated by epicutaneously tolerized mice (Fig 3, A). In contrast, CD8<sup>+</sup> T cells obtained from sensitized control mice secreted high amounts of IFN- $\gamma$  and IL-2 and only negligible or nondetectable amounts of T<sub>H</sub>2 cytokines, demonstrating the development of T<sub>H</sub>1 effector T cells of CHS (Fig 3, A).<sup>4</sup>

As demonstrated previously, after elicitation of CHS, CD4<sup>+</sup> T cells secrete IL-4, IL-10 and low levels of IFN- $\gamma$ , characterizing CD4<sup>+</sup> T cells as regulatory T cells of CHS (Fig 3, B).<sup>4</sup> An analysis of the cytokine pattern of CD4<sup>+</sup> T cells obtained from mice tolerized by epicutaneous, oral, or intravenous application revealed the production of low levels of IFN- $\gamma$  and no detectable or small amounts of the T<sub>H</sub>2 cytokines IL-4 and IL-10, excluding CD4<sup>+</sup> T cells as T<sub>H</sub>2 effector T cells of LZT (Fig 3, B).

#### Inhibited proliferation of T<sub>H</sub>1 CHS cells after oral and intravenous tolerization

Along the same line, LNC obtained from mice treated orally or intravenously with subimmunogenic doses of

TNBS before sensitization both displayed strongly reduced proliferative responses on hapten-specific restimulation *in vitro* compared with vigorously proliferating LNC obtained from sensitized control animals (Fig 3, B). Taken together, our results provide evidence that hapten-specific LZT suppressor CD8<sup>+</sup> T cells are generated after oral or intravenous administration of subimmunogenic hapten doses inhibiting the activation and proliferation of T<sub>H</sub>1 effector T cells of CHS.

#### CD8<sup>+</sup> suppressor T<sub>H</sub>2 cells are effector T cells in oral and intravenous tolerance to low doses of allergens

To this point, our data indicate that the cellular interactions of LZT evoked by different application routes and systemic distribution of low doses of haptens may represent a general mechanism of acquired peripheral tolerance. To verify whether systemically induced hapten-specific CD8<sup>+</sup> T<sub>H</sub>2 cells are the effector T cells of LZT preventing the development of CHS effector T cells, we transferred CD4<sup>+</sup> and CD8<sup>+</sup> T cells obtained from tolerized mice

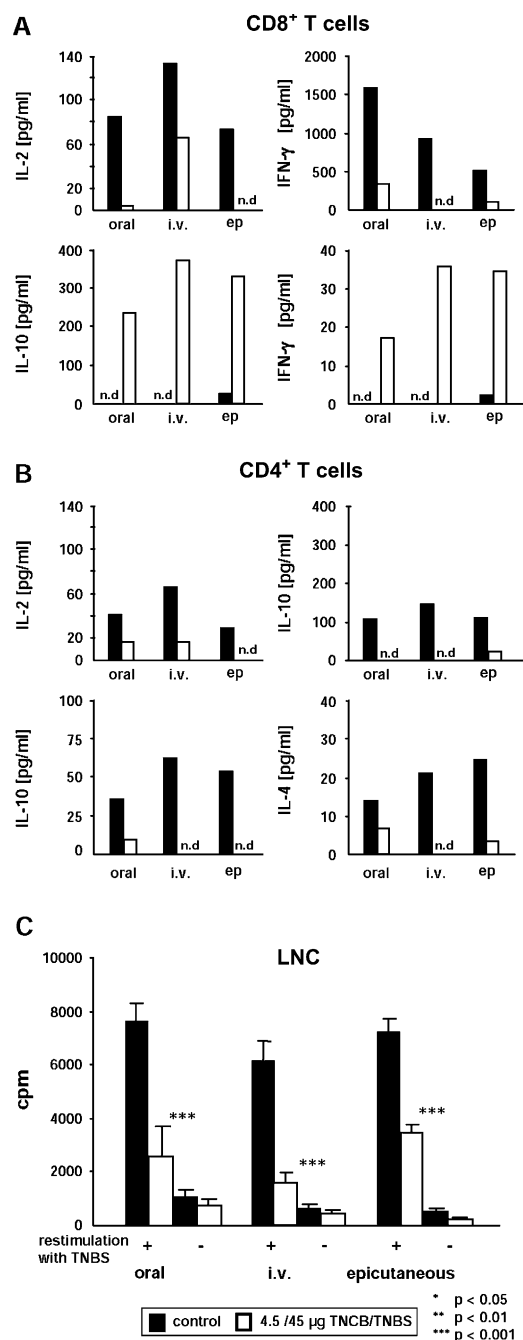


**FIG 3.** Development of CD8<sup>+</sup> T<sub>C2</sub> cells is induced in oral and intravenous tolerance to low doses of haptens. Mice were tolerized orally, intravenously, or epicutaneously as described. Twenty-four hours after challenge, lymph nodes were harvested, and whole LNC, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells were hapten-specific restimulated. Production of IFN-γ, IL-2, IL-4, and IL-10 by CD8<sup>+</sup> (A) and CD4<sup>+</sup> (B) was determined by ELISA. C, Proliferation of LNC, demonstrated as cpm ± SD, was assessed. n.d., Not detected; i.v., intravenous.

into naive recipients that subsequently were sensitized and challenged as described.

Transfer of CD8<sup>+</sup> T cells obtained from intravenously or orally tolerized mice induced a significant tolerance reaction in recipients as observed by reduced ear swelling,

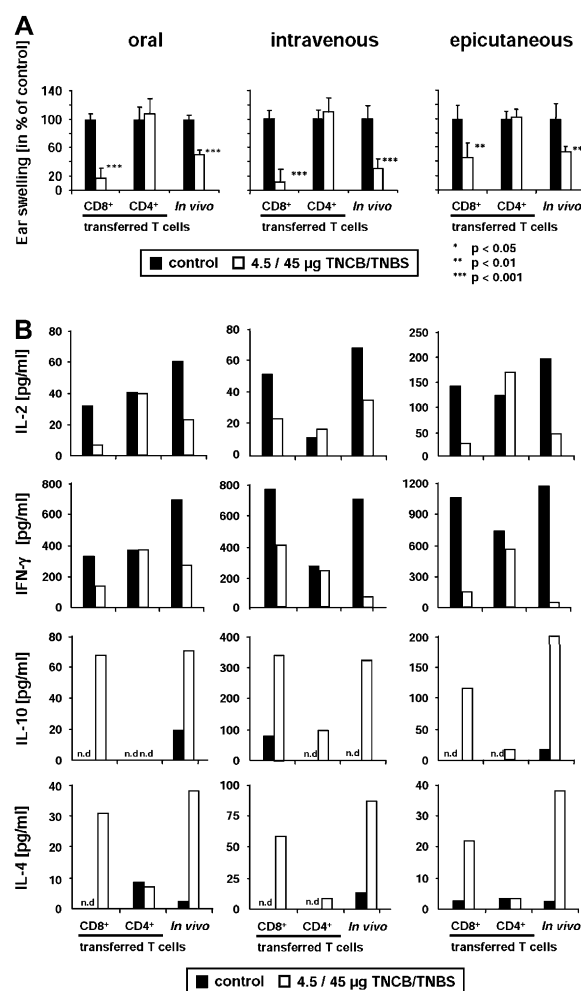


**FIG 4.** CD8<sup>+</sup> effector T<sub>C2</sub> cells of LZT impede the development of CHS. CD8<sup>+</sup> and CD4<sup>+</sup> subpopulations were purified from LNC of tolerized and control mice and injected into naive recipients. After sensitization and challenge (A), increase of ear thickness was measured (in % of sensitized controls ± SD). Mice without adoptive transfer of T cells served as *in vivo* controls. B, Cytokine concentration of CD8<sup>+</sup> T cells was detected by ELISA. n.d., Not detected.

demonstrating the generation of CD8<sup>+</sup> suppressor T cells of LZT after systemic distribution of the allergen (Fig 4, A). In contrast, an unrestricted CHS reaction was assessed compared with sensitized control mice if CD4<sup>+</sup> T cells were injected into naive mice, independent of an epicutaneous, oral, or intravenous treatment of the donor mice (Fig 4, A).

These findings were confirmed by *in vitro* proliferation assays. Only LNC obtained from recipients injected with CD8<sup>+</sup> T cells from epicutaneously as well as orally and intravenously tolerized donors all displayed markedly diminished proliferative responses compared with LNC from control sensitized mice or mice injected with CD4<sup>+</sup> T cells (data not shown).

In addition, we found that CD8<sup>+</sup> T cells obtained from experimental groups injected with CD8<sup>+</sup> T cells of tolerized mice exhibited markedly reduced IFN-γ release and greatly increased IL-10 and IL-4 secretion compared

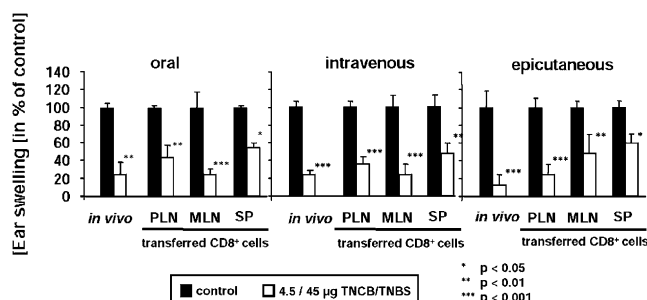


**FIG 5.** CD8<sup>+</sup> effector T cells of LZT are generated by systemic distribution of haptens. Tolerization protocols were performed as described. Peripheral skin draining lymph nodes (PLN), mesenteric lymph nodes (MLN), and spleens (SP) were taken, and purified CD8<sup>+</sup> T cells were injected into naive recipients. Mice without adoptive transfer of T cells served as *in vivo* controls. All animals were sensitized and challenged, and increase of ear thickness was assessed (in % of sensitized controls  $\pm$  SD).

with control sensitized mice (Fig 4, B). The levels of cytokines are comparable to amounts detected in control experiments performed with *in vivo* (without adoptive transfer of T cells) tolerized animals (Fig 4, B). In contrast, CD8<sup>+</sup> T cells from mice reconstituted with CD4<sup>+</sup> T cells obtained from tolerized animals released high amounts of IFN- $\gamma$  and IL-2 as found for sensitized control mice, demonstrating the development of a CHS-specific Tc1 cytokine pattern (Fig 4, B).

### CD8<sup>+</sup> effector T cells of LZT are widely distributed among peripheral lymphatic organs

Next, we performed adoptive transfer experiments with CD8<sup>+</sup> T cells of tolerized mice obtained from peripheral skin-draining lymph nodes or mesenteric lymph nodes or spleens to exclude that the route of tolerization governs the site of generation and stimulation of hapten-specific suppressor CD8<sup>+</sup> T cells of LZT. Experiments revealed that after oral, intravenous, or epicutaneous application of tolerizing amounts of the allergen, mesenteric skin-draining lymph nodes and spleen cells are found to be the source of CD8<sup>+</sup> effector T cells inhibiting the manifestation of CHS (Fig 5). *In vitro* analysis of the T-cell response (proliferation, cytokine pattern) of tolerized mice supported our *in vivo* observation and showed that CD8<sup>+</sup> effector Tc2 cells are induced in regional and distant, skin-draining, and nonskin-draining lymph nodes independent of the route of application (data not shown). These results indicate that the systemic application and distribution of haptens induce the development of CD8<sup>+</sup> effector T cells of LZT.

### Kinetics of oral and intravenous LZT

The kinetics underlying the development of tolerance was examined by performing the subsequent active sensitization after weekly intervals. It was found that after a single application (epicutaneous, oral, intravenous), the suppressive effect was significant as long as 3 weeks (Fig 6). When the time interval was prolonged up to 4 weeks, the tolerance effect had meanwhile faded. Increased numbers of applications (5 $\times$ ) of tolerizing doses every other day extended the tolerance state to as long as 5 weeks after the last application (Fig 6).

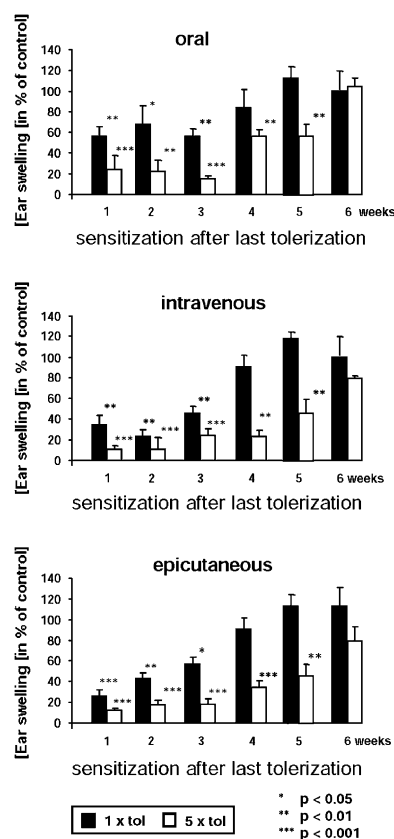


**FIG 6.** Kinetics of LZT after oral and intravenous induction. Mice were treated epicutaneously, orally, or intravenously with a single or 5 tolerizing doses of TNCB/TNBS. At the dedicated time points, sensitization was performed and tolerance development was tested as described. Increase of ear thickness was determined (in % of sensitized controls  $\pm$  SD).

### DISCUSSION

In this study, we demonstrate that LZT to allergens represents a systemic state of peripheral tolerance preventing the development of CHS-promoting Tc1 cells. The systemic application and distribution of haptens after epicutaneous, oral, and intravenous administration of

low amounts of allergens induced the sequential activation of regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations that inhibit the generation of effector T<sub>H</sub>1 T cells of CHS. The exposure to low doses of allergens is a physiologically and repeatedly occurring process that takes place by contact of allergens to the skin or mucosae of an organism. Therefore, LZT to haptens (eg, contact allergens, drugs, metals) may be an important mechanism of peripheral tolerance, preventing the development of allergic diseases. The report of a nickel-specific tolerance in nonallergic, healthy persons mediated by CD4<sup>+</sup>/CD25<sup>+</sup> T cells as a mechanism of naturally occurring tolerance is in line with our model of systemic tolerance induced by frequent exposure to allergens.<sup>22</sup>

In the past, different models of peripheral tolerance to contact allergens have been investigated, such as tolerance induced by UV-B irradiation before allergen application and intravenous injection of high doses of haptens.<sup>17,18,23,24</sup> A common feature of all those tolerance reactions is that epidermal Langerhans cells or dermal antigen-presenting cells are not activated but rather circumvented, as also demonstrated for LZT to contact allergens.<sup>8</sup> The mechanisms of tolerance induction by intravenous administration of contact allergens as antigens are even less understood, and there are only a few reports on that subject with respect to allergens.<sup>23,24</sup> Single injections of high, supraimmunogenic doses of haptens have been reported to induce a significant tolerance to subsequent epicutaneous sensitization with this contact allergen but the underlying immune reaction has not been analyzed.<sup>23,24</sup>

We also performed experiments by using high amounts (subimmunogenic doses  $\times 10^2$ - $10^4$ ) of the hapten. In contrast with intravenously induced LZT, injection of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells transferred this high zone tolerance into naive recipients (data not shown). These results suggest a different mechanism of tolerance to high amounts of haptens (high zone tolerance) compared with LZT to allergens. However, the data presented in this report provide strong evidence that identical immune reactions and regulatory T-cell subpopulations are involved in the process of LZT to haptens after systemic distribution of allergens applied epicutaneously as well as orally or intravenously.

The classic model of acquired peripheral tolerance is the oral tolerance to protein antigens.<sup>9,10</sup> Generally, a single high dose of protein antigen can induce either antigen-specific T-cell inactivation (anergy) or clonal deletion of antigen specific T cells.<sup>11,12</sup> However, feeding low doses of protein antigen can also induce oral tolerance, mediated by regulatory CD4<sup>+</sup> T-cell populations including Tr1 cells, which release large amounts of IL-10, and T<sub>H</sub>3 cells, which secrete predominantly TGF- $\beta$ .<sup>11,13-15</sup> In contrast, in the murine model of oral tolerance to nickel that is mediated by invariant natural killer T cells (iNKT) and regulatory CD4<sup>+</sup> T cells, IL-10 is not required for the induction of tolerance.<sup>21,22</sup> In contrast, as shown in this study in systemically induced LZT, IL-10-secreting, regulatory CD4<sup>+</sup> T cells evoke the generation of CD8<sup>+</sup> suppressor

T cells, preventing the induction of CHS. The differences in the T-cell immune response between our model of oral LZT to haptens and the system of protein-induced oral LZT may be a result of the features of the triggering antigen. It was reported that oral tolerance to protein antigens such as ovalbumin depends on the presence of Peyer patches, whereas, interestingly, oral tolerance to haptens was independent from the latter.<sup>25</sup>

Moreover, models of oral tolerance to contact allergens resulting in prevention or significant attenuation of CHS outcome have also emerged very early in literature. Among the contact allergens that can induce oral tolerance in CHS models are 2,4-dinitro-1-fluorobenzene (DNFB)<sup>26,27</sup> and TNCB.<sup>28</sup> In more recent studies, oral administration of DNFB has been reported to induce CD4<sup>+</sup> T cells, which are critical for the induction but not for the effector phase of tolerance to contact allergens.<sup>20</sup> Other authors consider CD4<sup>+</sup>CD25<sup>+</sup> T cells mandatory for induction of oral tolerance to haptens but exclude a role for IL-10 in their model.<sup>21</sup> In line with these results, in the murine model of oral tolerance to nickel that is mediated by iNKT and regulatory CD4<sup>+</sup> T cells, IL-10 is not required for the induction of tolerance.<sup>29,30</sup> In contrast, in our model of LZT, CD8<sup>+</sup> T cells are required as effector T cells of LZT. However, in all of these studies reported, contact allergens were used in high concentration (300-3000  $\mu$ g of various allergens) compared with our protocol of LZT to haptens. These data indicate that the development of different mechanisms of oral tolerance to contact allergens is a result of the amount of haptens (high versus LZT).

Taken together, our data point to LZT to haptens (eg, contact allergens, drugs) as a general mechanism of acquired peripheral tolerance induced by systemic uptake of small amounts of soluble hapten-antigens that naturally occurs under regular environmental circumstances. In contrast with other models of tolerance (oral tolerance to protein antigens or high zone oral tolerance to contact allergens), the LZT to contact allergens is mediated by regulatory IL-10 producing CD4<sup>+</sup> T cells and suppressor CD8<sup>+</sup> T<sub>H</sub>2 cells that impede the development of an allergic skin inflammation. The process of LZT to haptens may be critical for the prevention of unwanted activation of the immune system and protection of the organism from allergies and other immune-mediated diseases.

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