Fas-Mediated Inhibition of CD4+ T Cell Priming Results in Dominance of Type 1 CD8+ T Cells in the Immune Response to the Contact Sensitizer Trinitrophenyl

Stefan F. Martin, Jan C. Dudda, Virginie Delattre, Eva Bachtanian, Cornelia Leicht, Beate Burger, Hans Ulrich Weltzien and Jan C. Simon

*J Immunol* 2004; 173:3178-3185; doi: 10.4049/jimmunol.173.5.3178

http://www.jimmunol.org/content/173/5/3178

References

This article cites 49 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/173/5/3178.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Fas-Mediated Inhibition of CD4⁺ T Cell Priming Results in Dominance of Type 1 CD8⁺ T Cells in the Immune Response to the Contact Sensitizer Trinitrophenyl

Stefan F. Martin, Jan C. Dudda, Virginie Delattre, Eva Bachtanian, Cornelia Leicht, Beate Burger, Hans Ulrich Weltzien, and Jan C. Simon

One of the unusual properties of chemically reactive haptens is their capacity to simultaneously generate immunogenic determinants for hapten-specific CD8⁺ and CD4⁺ T cells. Surprisingly, however, a clear dominance of CD8⁺ effector T cells is observed in murine contact hypersensitivity to various haptens and upon T cell priming with hapten-modified APCs in vitro. In this study we show that trinitrophenyl-specific CD8⁺ T cells actively prevent CD4⁺ T cell priming in vitro. This process requires cell-cell contact and is dependent on the expression of Fas on the CD4⁺ T cells. Our results reveal an important Fas-dependent mechanism for the regulation of hapten-specific CD4⁺ T cell responses by CD8⁺ T cells, which causes the dominance of CD8⁺ effector T cells and the active suppression of a CD4⁺ T cell response. Moreover, our demonstration of reduced contact hypersensitivity to trinitrophenyl in the absence of Fas, but not of perforin and/or granzymes A and B, underlines the important role of Fas as a pathogenetic factor for contact hypersensitivity.


Hapten-induced contact hypersensitivity (CHS) is a T cell-mediated immune response. Recent studies have shown that the type 1 CD8⁺ T cell subset (Tc1) is the dominant effector population in trinitrophenyl- (TNP), DNP-, and oxazolone-induced CHS in C57BL/6 and BALB/c mice (1–5). These T cells exert cytotoxicity toward hapten-modified target cells, e.g., epidermal keratinocytes (6–8), by means of the Fas and perforin (perf) pathways. Both cytolytic mechanisms seem to be required for the efficient induction of CHS (9). CD8⁺ T cells have also been shown to be important effectors in allergenic responses to metal ions and drugs (10–12) as well as in other allergies, asthma, and autoimmune responses (13). CD4⁺ regulatory T cells have been shown to down-regulate the CHS response (1, 2).

A clear dominance of cytotoxic, IFN-γ-producing CD8⁺ T cells is also observed when spleen or lymph node cells are primed with TNP in vitro. Induction of CD4⁺ T cells specific for TNP does not occur unless CD8⁺ T cells are depleted (14–16).

The dominance of CD8⁺ effector T cells in CHS responses to experimental haptens (1–5) and to TNP in vitro (14–16) is explained to date. An efficient activation of CD4⁺ and CD8⁺ T cells by chemical haptens should be expected, because protein-reactive molecules such as TNP or penicillin can simultaneously generate antigenic determinants for both T cell subsets. Direct covalent modification of peptides associated with MHC class I and MHC class II molecules on the surface of APCs can be envisaged as well as modification of intra- or extracellular proteins, which are then processed by APC to yield MHC class I- or MHC class II-bound haptenated peptides (17–20).

The goal of this study was elucidation of the potential mechanisms responsible for the observed dominance of CD8⁺ and the absence of CD4⁺ effector T cells in TNP-induced immune responses in C57BL/6 (B6) mice. We demonstrate that TNP-specific cytotoxic CD8⁺ Tc1 effectors are rapidly induced in vivo and in vitro. CD8⁺ T cells prevent priming of CD4⁺ T cells via a Fas (CD95)-mediated, perf-independent induction of apoptosis in vitro. Fas-deficient mice mounted a significantly reduced CHS response to trinitrophenyl, whereas the absence of perf and/or granzymes (gzm) did not impair CHS. Besides the role of Fas in the regulation of CD4⁺ T cell responses to TNP by CD8⁺ T cells in vitro, the latter findings support the role of Fas as an important pathogenetic factor for eczematous skin diseases (21).

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) or were provided by the breeding facility of Max Planck Institute for Immunobiology (Freiburg, Germany). Perf knockout (KO) mice (22), Fas mutant lpr mice (B6.1pr), B6.Fas KO (23), gzmB KO (24), gzmA×B KO (25), and perf×gzmB and perf×gzmA×B KO C57BL/6 mice (26) were gifts from Dr. M. M. Simon (Max Planck Institute for Immunobiology). Mice were used at the age of 5–8 wk. All experimental procedures were in accordance with Max Planck Institute and University of Freiburg guidelines on animal welfare.

Media and chemicals

RP-10 consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES buffer, 50 μg/ml penicillin-streptomycin (all from Invitrogen Life Technologies, Eggenstein, Germany), and 10 μM 2-ME (Sigma-Aldrich, Deisenhofen, Germany). Trinitrobenzene sulfonic acid (TNBS) was obtained from Sigma-Aldrich, and trinitrochlorobenzene (TNCB) was obtained from VeZet Laborsynthesen (Idar-Oberstein, Germany).
Antibodies
Anti-CD4 (GK1.5), anti-CD8α (53-6.7), anti-CD11c (HL3), anti-CD62L (MEL-14), anti-CD3 (M384), anti-GR1 (RB6-8C5), anti-I-A<sup>+</sup> (AF6-120.1), anti-CD95 (Jo2), anti-CD178.1 (Kay-10), and isotype control Abs were all from BD Pharmingen (Heidelberg, Germany) and were used as FITC, PE, or biotin conjugates. Biotinylated Abs were revealed by streptavidin-CyChrome (BD Pharmingen). Anti-IL-4 (11B11, BV6-24G2) and anti-IFN-γ (R4-6A2, XMG1.2) were obtained from BD Pharmingen and used purified or biotinylated.

Generation of bone marrow-derived dendritic cells
Bone marrow was collected from the tibia and femur of 6- to 8-wk-old C57BL/6 mice. A single-cell suspension was prepared, and erythrocytes were lysed. After repeated washes, bone marrow cells were plated at 1 × 10<sup>5</sup>/well in 24-well plates in RP-10 in the presence of 40 ng/ml GM-CSF culture supernatant and 10 ng/ml rIL-4 (Promocell, Heidelberg, Germany). Cultures were fed with fresh medium containing GM-CSF and IL-4 on days 3 and 5 by replacing half the medium. On day 6, cells were harvested and isolated over a 14.5% metrizamide (Serva, Heidelberg, Germany) gradient. After repeated washes in PBS, cells were resuspended and used for immunization or for in vitro experiments. The quality of the dendritic cell (DC) preparation was controlled by three-color flow cytometry. Cells were typically CD11c<sup>+</sup>, Mac-3<sup>-</sup>, GR-1<sup>-</sup>, MHC class II I-A<sup>+</sup> high.

In vitro priming of T cells with dendritic cells
Cultures were set up in 24-well plates using 5 × 10<sup>5</sup> TNP-modified DC and a mixture of 2 × 10<sup>5</sup> spleen/lymph node (LN) cells/well. The cultures were restimulated after 7 days with fresh TNP-modified DC. DC were modified by incubation with 3 mM TNBS for 7 min at 37°C, followed by three washes with RP-10.

Immunization and induction of contact hypersensitivity
Mice were sensitized by two intracutaneous (i.c.) injections of 3 × 10<sup>5</sup> DC into two sites of the shaved abdomen in 200 μl of PBS as previously described (4, 5, 27), followed by epicutaneous application of 3 mM TNBS for 7 min on the dorsum of both ears 5 days later. Alternatively, 100 μl of 7% TNCB/acetone was painted on the shaved abdomen for sensitization, followed by elicitation as described above. Ear thickness was measured before and 24 h after ear challenge using an engineer’s micrometer (Mitutoyo, Leonberg, Germany).

In vitro stimulation after CHS
Auricular, maxillary and axillary lymph nodes were pooled 24 h after TNCB ear challenge. A single-cell suspension was prepared. Erythrocytes were lysed, and 4 × 10<sup>5</sup> cells/well were cocultivated with 3 × 10<sup>5</sup> irradiated (3000 rad) C57BL/6 spleen cells modified with 3 mM TNBS for 7 min at 37°C as previously described (4). Cultivation was performed in 96-well, U-bottom plates (Costar, Cambridge, MA). Cultures were harvested after 36 h for ELISA supernatants or were used at the indicated time points as effectors in cytotoxicity assays. For ELISPOT assays, cultures were set up in ELISPOT plates, and assays were developed after 36 h (5).

ELISA
ELISA was performed as previously described (5). Briefly, plates (Greiner, Nurtingen, Germany) were coated with anti-IFN-γ or anti-IL-4 capture Abs (BD Pharmingen) at 4°C overnight. After repeated washes and blocking, recombinant cytokine standards (BD Pharmingen) and samples (culture supernatant) were diluted. The assay was developed after washing, using biotinylated detection Abs (BD Pharmingen) and streptavidin-coupled HRP (Dianova, Hamburg, Germany). Substrate (3,3′,5,5′-tetrachloro-3-aminophenol and 4-nitrophenylacetate) was added for 10 min in the dark, and the reaction was stopped by addition of 0.5 M H<sub>2</sub>SO<sub>4</sub>. OD at 405 nm was measured in a microplate reader (MR 5000; Dynatech Laboratories, Denkendorf, Germany), and cytokine concentrations were calculated using BioLine software (Dynex Technologies, Denkendorf, Germany).

ELISPOT assay
ELISPOT assay was performed as previously described (5). Briefly, plates (Multiscree HA, 96-well plates; Millipore, Eschborn, Germany) were coated with anti-IFN-γ (R4-6A2; 10 μg/ml in PBS) or anti-IL-4 (11B11; 5 μg/ml in PBS) overnight, washed, and blocked for 1 h at 37°C by addition of RP-10. Medium was discarded, and 2-fold dilutions of 2 × 10<sup>5</sup> LN cells/well in RP-10 were added. Irradiated (10,000 rad) MHC class II-positive JAWSII dendritic cells (CRL-11904; American Type Culture Collection, Manassas, VA) were added in 7 min at 37°C, washed repeatedly, and resuspended in RP-10 supplemented with 5% rat Con A-induced rat spleen cell supernatant/1% α-methylmannoside (Sigma-Aldrich), and added to the wells. The assay was incubated for 36 h at 37°C in 5% CO<sub>2</sub>. Cells were then discarded, biotinylated anti-IFN-γ (XMG1.2; 5 μg/ml) or anti-IL-4 (BV6-24G2; 5 μg/ml in PBS/2% FCS) was added (100 μl/well), and plates were incubated overnight at 4°C. After repeated washes streptavidin-conjugated HRP (Dianova) was added for 1 h at room temperature. The assay was developed by addition of substrate (1 mg/ml diaminobenzidine (Sigma-Aldrich) in 50 mM Tris, pH 7.5) after mixing with 1 μM 30% H<sub>2</sub>O<sub>2</sub>. Plates were rinsed with water 10–15 min later to stop the reaction. Spots were counted microscopically using the KS ELISPOT system (Zeiss Vision, Hallbergmoos, Germany). Frequencies were determined using wells at cell concentrations yielding well-separated cytokine spots. The corrected frequencies of cells producing cytokines in a TNP-specific manner were calculated by subtracting the mean spot number of duplicate cultures stimulated with unmodified APC from the mean spot number of duplicate cultures stimulated with TNP-modified APC. The absolute number of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the respective wells was calculated based on ex vivo flow cytometric quantitation of the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the lymph nodes.

Magnetic bead depletion
CD4<sup>+</sup> or CD8<sup>+</sup> T cells were depleted from total LN cells using anti-mouse CD4 or CD8 Dynabeads (Dynal Laboratories) according to the manufacturer’s protocol. DC were depleted using biotinylated anti-CD11c and streptavidin-conjugated beads.

Flow cytometry
Cells (5 × 10<sup>5</sup> to 1 × 10<sup>6</sup>) were stained for 20 min with 0.5–1 μg of biotinylated mAb in 100 μl of PBS/2% FCS/0.02% Na<sub>2</sub>SO<sub>3</sub> (FACS buffer) on ice. Cells were washed three times in FACS buffer, and FITC- and PE-conjugated secondary Abs as well as streptavidin-CyChrome (BD Pharmingen) were added for 20 min on ice. Samples were washed three times and resuspended in 200 μl of 1% paraformaldehyde. Data were acquired on a FACScan instrument (BD Biosciences, Heidelberg, Germany) and analyzed using the CellQuest software (BD Biosciences).

In vitro cell cycle analysis, cells (5 × 10<sup>5</sup> to 1 × 10<sup>6</sup>) were stained with FITC-conjugated anti-CD4 or anti-CD8α, washed, and resuspended in 500 μl of FACS buffer. Cold 70% ethanol (1.5 ml) was added. Samples were gently vortexed and incubated for 45 min at 4°C. Cells were spun for 5 min at 496 × g and resuspended in 250 μl of FACS buffer. Ten microliters of RNase A (1 mg/ml; Roche, Mannheim, Germany) and 12.5 μl of propidium iodide (1 mg/ml, Sigma-Aldrich) were added, and cells were incubated for 30 min in the dark at room temperature, washed, resuspended in FACS buffer, and analyzed.

Cytotoxicity assays
EL4 lymphoma cells were labeled with Na<sup>21</sup>CrO<sub>4</sub> (Amersham Biosciences, Freiburg, Germany) for 90 min, washed three times, and modified with 3 mM TNBS or left untreated. For peptide pulse, target cells were incubated with 5 μM peptide during labeling and washed three times. CTL from T cell cultures were harvested and washed repeatedly, and 3-fold serial dilutions were made. Target cells were added, and plates were spun for 5 min at 51 × g. The assay was then incubated for 4 h at 37°C in 5% CO<sub>2</sub>. Plates were spun for 5 min at 296 × g, and 30 μl of supernatant was transferred to Luma plates (PerkinElmer, Rodgau-Jugesheim, Germany). Counts were measured in a TopCount (PerkinElmer), and the percent specific lysis was calculated using RIASmart software (PerkinElmer).

Statistics and statistical analysis
All individual experiments shown are representative of a minimum of three independent experiments. Statistical analysis was conducted using t test. Differences were statistically significant at p < 0.05.

Results
Preferential induction of TNP-specific, IFN-γ-producing, and cytotoxic CD<sup>+</sup> T cells in CHS
Mice were sensitized by i.c. injection of unmodified or TNP-modified DC. CHS was elicited 5 days later by ear challenge with TNCB. This immunization route or sensitization by skin painting with TNCB efficiently induces skin homing CD<sup>+</sup> T cells (28).
TNP-specific ear swelling was detected 24 h after injection of TNP-modified, but not unmodified, control DC (Fig. 1A).

Auricular, maxillary, and axillary LN cells were pooled 72 h after ear challenge and restimulated in vitro with unmodified or TNP-modified JAWS II DC for 36 h (ELISPOT, Fig. 1B) or with TNP-modified spleen cells for 72 h (CTL assay, Fig. 1C, flow cytometry, Fig. 2). ELISPOT assay revealed TNP-specific IFN-γ production (Fig. 1B) and the absence of IL-4 (not shown). These results were confirmed by ELISA (not shown). Furthermore, for identification of the IFN-γ-producing T cell subset, we depleted CD4+ or CD8+ T cells from the LN cell population. IFN-γ production was unaffected upon depletion of CD4+ T cells, whereas it was completely abrogated in the absence of CD8+ T cells (Fig. 1B).

The percentages of CD4+ and CD8+ T cells in LN were determined ex vivo by flow cytometry to allow for calculation of the frequency of TNP-specific, IFN-γ-producing T cells in C57BL/6 mice. The frequency of TNP-specific, CD8+ T cells determined from the undepleted or CD4-depleted LN cell population used in the assay was roughly 0.5% of all CD8+ T cells (5).

TNP-specific cytotoxic activity was detected in LN from mice sensitized with DC-TNP, but also in LN sensitized with control DC (Fig. 1C). The latter received only a single ear challenge with TNCB. This demonstrates the efficiency of a single hapten application for rapid induction of TNP-specific Tc1 effectors (5).

![FIGURE 1](image1.png)

**FIGURE 1.** Dominance of CD8+ Tc1 effector cells in TNP-induced CHS. A, Mice were sensitized by i.c. injection of untreated control (DC) or TNP-modified DC (DC-TNP). CHS was elicited 5 days later by challenge with TNCB on both ears. Values represent the mean 24-h ear swelling of three mice ± SD (p < 0.001). B, Seventy-two hours after TNCB ear challenge, pooled auricular, maxillary, and axillary LN cells were restimulated for 36 h in vitro with unmodified (no Ag) or TNP-modified (TNP) JAWS II DC. LN cells were used undepleted or were depleted of CD4+ or CD8+ T cells for ELISPOT. Spots were counted microscopically. The values represent the mean of duplicate measurements. C, A 4-h CTL assay was performed with LN cells after TNCB ear challenge and 72-h in vitro restimulation with TNP-modified spleen cells. EL4 lymphoma cells were used as target cells and were either unmodified or TNP-modified. The E/T cell ratio is indicated. Numbers indicate the percent specific lysis.

The activation of T cell subsets by TNP in vivo was also monitored by flow cytometry of the LN cells 72 h after restimulation in vitro. Analysis of the T cell activation marker CD62L revealed a strong increase in the population of activated CD62Llow CD8+ T cells in cultures from DC-TNP-immunized mice compared with cultures from control DC-immunized mice after CHS elicitation by ear challenge with TNCB (from 11.2 to 37.8%; Fig. 2). No significant differences between control mice and DC-TNP-immunized mice were seen with ex vivo LN cells (not shown). Interestingly, no efficient activation of CD4+ T cells was detectable (from 9.4 to 12.2%). Similarly, after sensitization of mice by TNCB painting, instead of i.c. DC-TNP injection and elicitation of CHS, we observed a selective increase in activated CD8+ T cells compared with acetone-painted control mice (from 2.4 to 20.3%), again with an absence of CD4+ T cell activation (from 6.4 to 8.3%; data not shown). These results are in line with the cytokine and cytotoxicity data (Fig. 1, B and C), demonstrating a lack of TNP-specific CD4+ T cells.

**CD8+ T cells actively suppress CD4+ T cell priming**

We used an in vitro culture system for the analysis of a potential influence of CD8+ effector T cells on the priming of CD4+ T cells. Priming of naive, undepleted C57BL/6 spleen and LN cells with TNP-modified DC resulted in efficient and selective activation of CD8+ T cells and an absence of CD4+ T cell priming (Fig. 3). CD8+ T cell priming was also efficient in the absence of CD4+ T cells. In sharp contrast, only when CD8+ T cells were depleted did we observe a very efficient priming of CD4+ T cells by the TNP-modified DC (74 vs 8% CD62Llow CD4+ T cells). These results clearly indicate that the lack of CD4+ T cell priming in the presence of CD8+ T cells is due not to a lack of MHC class II-dependent TNP presentation, but to an active suppression by TNP-specific CD8+ T cells.

To analyze the role of cell-cell contact for the observed inhibition of CD4+ T cell priming, we used a Transwell culture system (data not shown). Efficient priming of CD4+ T cells by DC-TNP in the presence of CD8+ T cells was observed when the latter were primed in a tissue culture insert. Therefore, direct cell-cell contact between CD4+ and CD8+ T cells is required for the active suppression of CD4+ T cell priming.
Efficient induction of TNP-specific CD4+ T cells in the absence of CD8+ T cells

To characterize the subset of CD4+ T cells induced by TNP, we primed CD8+ T cell-depleted spleen/LN cells with DC-TNP in vitro. Flow cytometry (Fig. 4A) and measurement of cytokines (Fig. 4B) produced in CD8+ T cells as a control, CD4+ T cell-depleted cultures revealed the efficient induction of CD62Llo/CD8α+ (upper panels) or CD4+ T cells (lower panels).

Rapid induction of TNP-specific CTL effectors in vitro

To test whether suppression of CD4+ T cell priming by CD8+ T cells was caused by cytotoxic mechanisms of the rapidly generated CD8+ effector T cells, spleen and LN T cells from unimmunized C57BL/6 mice were cocultured with TNP-modified DC or unmodified control DC. Flow cytometry for CD4, CD8, and CD62L was performed every day to monitor the priming of effector T cells (Fig. 5A). Cytotoxicity assays were performed on days 3 (not shown) and 4. No activation was detectable in the CD4+ T cell compartment, as determined by the generation of CD62Llo T cells, whereas priming of CD8+ T cells was observed as early as day 3 (Fig. 5A). A burst of CD62Llo CD8+ T cells was detected between days 3 and 4, and this correlated with robust TNP-specific CTL activity on day 4 (Fig. 5B). This rapid induction of TNP-specific CTL was also observed after a single hapten application in vivo (Fig. 1C) and may be due to the high frequency of TNP-specific CD8+ T cells in C57BL/6 mice (5) and the potent antigenicity of TNP.

Suppression of CD4+ T cell priming by CD8+ T cells is Fas mediated

We considered rapid cytolyis of APC or bystander cytolsis of CD4+ T cells as a potential mechanism for preferential CD8+ T cell priming. In our in vitro priming system, coculture of CD8+ T cells from perforated KO mice (22) with wild-type CD4+ T cells did not result in efficient CD4+ T cell priming compared with the undepleted control culture (Fig. 6A). In contrast, when Fas mutant CD4+ T cells from B6.lpr mice were used, efficient priming of these CD4+ T cells occurred even in the presence of wild-type CD8+ T cells (Fig. 6B). These results demonstrate that CD4+ T cell priming is prevented by the TNP-specific CD8+ effector T cells via Fas/FasL interaction. When DC from B6.lpr mice were used as APC, we did not observe a significant improvement of the efficiency of CD4+ T cell priming (data not shown). This suggests that APC cytolsis by the primed CD8+ T cells resulting in an insufficient stimulation of CD4+ T cells does not play a role in the lack of CD4+ T cell priming.

Preferential apoptosis of CD4+ T cells in the presence of CD8+ T cells

Because B6.lpr CD4+ T cells are resistant to CD8+ T cell-mediated suppression, we analyzed the expression of Fas (CD95) and FasL (CD178) on CD4+ T cells (Fig. 6). The percent-aggs of activated CD62Llo/CD8α+ (upper panels) or CD4+ T cells (lower panels).
Both T cell subsets expressed significant apoptosis of CD4+ T cells (Fig. 5) and their Fas-mediated suppression of CD4+ T cells (Fig. 6).

**Decreased CHS response in Fas-deficient mice**

Because Fas-mediated suppression of CD4+ T cell priming is responsible for the dominance of Tc1 responses to TNP in vitro, we reasoned that the absence of Fas may allow for a more efficient action of CD4+ IL-4/IL-10-producing regulatory T cells, which down-regulate CHS (1, 2). We induced CHS in Fas KO (23) instead of B6.lpr mice, because lpr mice mount a normal CHS response (9). In fact, we found that CHS was significantly decreased in Fas KO mice compared with wild-type B6 mice (Fig. 8A). Due to the lack of an increase in T cells secreting cytokines such as IL-4 or IL-10 (data not shown), these results can be interpreted in several ways. For example, there could be a shift in the balance of Tc1 effectors toward regulatory T cells, which is not detectable at the level of cytokine profiles, or the decreased CHS response may be due to the lack of Fas-mediated keratinocyte apoptosis. The latter is considered to be a crucial factor in the pathogenesis of CHS or atopic dermatitis (6–8, 21). The residual Fas activity due to the leaky phenotype of the lpr mutation (23) may therefore be responsible for the normal CHS response in B6.lpr in contrast to Fas KO mice.

**CHS is not impaired in mice deficient for perf and/or gzm**

Based on our results with Fas KO mice, we analyzed the roles of other mechanisms of T cell-mediated cytotoxicity (29) in the development of CHS. Granule exocytosis by T lymphocytes is believed to be involved in the pathogenesis of drug-induced exanthema (30, 31), atopic dermatitis, and psoriasis (32). Furthermore, the presence of perf and gzmB has been demonstrated in the skin of patients with drug-induced exanthema (31). We tested the contributions of perf and gzm to the allergic response, because it has been reported that perf KO C57BL/6 mice develop a normal CHS response to dinitrofluorobenzene (DNFB), but perf×lpr double-deficient mice do not develop an ear-swelling reaction despite induction of hapten-specific CTL in lymph nodes (9). We made use of mouse strains with gene KOs for perf and/or gzmA and -B (24–26). As shown in Fig. 8B, none of the mouse strains showed compromised CHS responses to TNP. In addition, gzmA (33) and perf×gzmA-deficient mice (26) mounted normal CHS responses (data not shown).

**Discussion**

In this study we have analyzed the potential factors responsible for the dominance of CD8+ Tc1 effector cells in the immune response to the hapten TNP as observed in the CHS model and in an in vitro priming system. A single hapten immunization rapidly and selectively induced Tc1 cells at high frequencies in vivo (5) and in vitro. Our results underline the strong antigenicity of TNP, which induces an efficient, CD4+ T cell-independent Tc1 response.

The selectivity of this T cell priming was unexplained to date. Our previous studies have shown that the efficient generation of TNP-specific CD4+ T cell lines was only possible if CD8+ T cells were depleted before in vitro priming with TNBS-modified spleen cells (14–16). In this study we demonstrate that in the absence of CD8+ T cells, IFN-γ-producing CD4+ T cells were efficiently primed in vitro using DC as APC. These data clearly show that the epitopes for priming of TNP-specific CD4+ T cells are efficiently generated when splenic APC or DC are modified with TNBS. Interestingly, we found that CHS is efficiently induced by TNCB in CD8+ T cell-deficient, β2-microglobulin KO mice, clearly demonstrating the ability of IFN-γ-producing CD4+ Th1 cells to induce this reaction in the absence of CD8+ T cells (J. C. Dudda,
B. Burger, E. Bachtanian, and S. F. Martin, manuscript in preparation), which is in line with our in vitro findings. Furthermore, in vivo Ab depletion of CD8 T cells does not abrogate CHS to TNCB in B6 mice (J. C. Dudda and S. F. Martin, unpublished observations) or to DNFB in BALB/c mice (34).

The simultaneous priming of CD4 and CD8+ T cells in a Transwell system clearly shows that cell-cell contact between CD4+ and CD8+ T cells is crucial for the suppression. Soluble factors do not seem to play a role (data not shown).

One possible explanation for our observations is a three-cell interaction, as also suggested to explain CD4 T cell help for CTL induction (35). We envisage a hapten-specific suppression of CD4+ T cells due to a simultaneous interaction of the DC, the TNP-specific CD8+ and the CD8+ T cell during priming in the lymph node. We have never observed fratricide of TNP-specific CD8+ T cells. Classical bystander cytolyis (36–38) of CD4+ T cells is the likely mechanism responsible for CD4+ T cell suppression. The prevention of CD4+ T cell priming is due to CD8+ T cell-mediated, Fas-dependent induction of apoptosis. When we analyzed the kinetics of preferential priming of CD8+ T cells by DC-TNP in vitro (Fig. 5A), we observed a parallel steady and selective increase in subdiploid CD4+ T cells up to day 4 of culture (not shown).

We have excluded a crucial role for rapid APC killing using Fas-deficient DC from B6.Lpr mice (data not shown). In this context, it is interesting to note that CD4+ and CD8+ T cells acquire significant amounts of TNP from DC-TNP in vitro or after skin painting with TNCB in vivo (J. C. Dudda, B. Burger, M. Averbeck, B. Kremer, C. Termeer, E. Bachtanian, and S. F. Martin, manuscript in preparation). Whether the presence of TNP on the CD4+ T cells is functionally relevant for the suppression by CD8+ T cells is currently under investigation.

It remains to be determined whether this in vitro mechanism is responsible for the in vivo dominance of CD8+ effector T cells. It is, however, tempting to speculate that CD4+ regulatory T cells that have been shown to down-regulate the response in hapten-induced CHS in mice by production of IL-4/IL-10 (1, 2, 39, 40) and in nickel-induced CHS in man (10, 41) could be suppressed initially by such a mechanism allowing for allergen sensitization. However, regulatory T cell function remains unaffected in the elicitation phase, because the CHS response is efficiently down-regulated. To date, it has been unclear whether regulatory T cells in our system are activated in the same LN at the same time as the CD8+ effector T cells or whether pre-existing regulatory T cells are directly recruited to inflamed skin from the circulation. It is also unknown whether they would be susceptible to Fas-mediated suppression. This is, however, unlikely, given their broad range of activities (42). Therefore, our observation that Fas KO mice mount significantly impaired CHS responses is most likely due to a lack of Fas-mediated killing of keratinocytes in the skin (6–8, 21). No contribution of perf and gzmB or gzmA to the development of TNP-mediated CHS has been found in our studies.

In contrast to the concomitant activation of CD4+ IL-4/IL-10-producing T cells and CD8+ Tc1 cells in BALB/c mice in response to DNFB or oxazolone (43), we only observed CD8+ Tc1 activation by TNP in B6 mice (4, 5). One explanation for this hapten-specific discrepancy may be different kinetics of regulatory T cell activation or recruitment in these two mouse strains.

In general, a wide variety of delayed-type hypersensitivity reactions that was previously assigned to CD4+ T cells turns out to be caused by CD8+ T cells, as reviewed recently (13). Many of these reactions are caused by chemical allergens and contact sensitizers such as TNP, which simultaneously generate MHC class I- and MHC class II-associated antigenic T cell determinants. The dominance of CD8+ T cell responses in these systems is not understood as yet, but may be due, for example, to the mechanisms described in this study. Further studies using, for example, Fas-deficient mice in the CHS model are needed to answer this question, but they are complicated due to the pleiotropic effects of the Fas/FasL system as an inducer of keratinocyte apoptosis in CHS (8, 9, 21), a suppressor of CD4+ T cell priming in vitro, and its dual function as an inducer of T cell apoptosis and a costimulatory system (44–46). Interestingly, a recent study reported that the predominance of Th2 cells in atopic disease is due to preferential apoptosis of Fas-expressing Th1 cells. Apoptosis was triggered in vivo, as shown, for example, by the formation of active caspase 8, which is involved in Fas-mediated cell death (47).
The importance of the type of T cell response (CD4 vs CD8) has been very impressively demonstrated in murine experimental allergic encephalomyelitis. A major role for cytotoxic CD8\(^{+}\) T cells in demyelination has been discovered (48, 49). A substantial contribution of CD8\(^{+}\) T cells to various autoimmune diseases is now recognized (50). In these studies, different forms of disease and outcomes of therapeutic approaches have been observed based on the subset of the pathogenic effector T cells. This also seems to be true for hapten-specific T cell responses such as adverse drug reactions. Thus, a special bular cutaneous manifestation of penicillin allergy is dominated by cytotoxic CD8\(^{+}\) T cells (11, 12).

In summary, we have defined a new role for CD8\(^{+}\) T cell-mediated cytotoxicity in the immune responses to the hapten TNP. An interesting mechanism for the regulation of CD8\(^{+}\) T cells by CD8\(^{+}\) T cells was found. Thus, TNP-specific, cytotoxic CD8\(^{+}\) T cells prevent a CD4\(^{+}\) effector T cell response by Fas-mediated induction of apoptosis in vitro. This results in the dominance of CD8\(^{+}\) Tc1 effector cells. It remains to be determined whether this mechanism is responsible for the in vivo dominance of CD8\(^{+}\) effector T cells in allergic reactions of mice to experimental haptenes (1–5, 13) and by humans to drugs such as \(\beta\)-lactam antibiotics (7, 8).

Acknowledgments
We thank Dr. Annalisia Lembo for carefully reading the manuscript. We also thank Dr. Markus M. Simon, and Thomas Stehle (Max Planck Institute for Immunobiology, Freiburg, Germany) for their generous supply of Fas-, perforin-, and granzyme-deficient mice.

References
2. Xu, H., N. A. Dilullo, and R. L. Fairchild. 1996. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon γ-producing (Tc1) effector CD8\(^{+}\) T cells and interleukin (IL)-4/IL-10-producing (Tc2) regulatory CD4\(^{+}\) T cells. J. Exp. Med. 183:1001.