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$CD4^+$ Th1 and $CD8^+$ Type 1 Cytotoxic T Cells Both Play a Crucial Role in the Full Development of Contact Hypersensitivity[1]

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The role of $CD4^+$ vs $CD8^+$ T cells in contact hypersensitivity (CHS) remains controversial. In this study, we used gene knockout (KO) mice deficient in $CD4^+$ or $CD8^+$ T cells to directly address this issue. Mice lacking either $CD4^+$ or $CD8^+$ T cells demonstrated depressed CHS responses to dinitrofluorobenzene and oxazolone compared with wild-type C57BL/6 mice. The depression of CHS was more significant in CD8 KO mice than in CD4 KO mice. Furthermore, in vivo depletion of either $CD8^+$ T cells from CD4 KO mice or $CD4^+$ T cells from CD8 KO mice virtually abolished CHS responses. Lymph node cells (LNCs) from hapten-sensitized CD4 and CD8 KO mice showed a decreased capacity for transferring CHS. In vitro depletion of either $CD4^+$ T cells from CD8 KO LNCs or $CD8^+$ T cells from CD4 KO LNCs resulted in a complete loss of CHS transfer. LNCs from CD4 and CD8 KO mice produced significant amounts of IFN-γ, indicating that both $CD4^+$ and $CD8^+$ T cells are able to secrete IFN-γ. LNCs from CD8, but not CD4, KO mice were able to produce IL-4 and IL-10, suggesting that IL-4 and IL-10 are mainly derived from $CD4^+$ T cells. Intracellular cytokine staining of LNCs confirmed that IFN-γ-positive cells consisted of $CD4^+$ (Th1) and $CD8^+$ (type 1 cytotoxic T) T cells, whereas IL-10-positive cells were exclusively $CD4^+$ (Th2) T cells. Collectively, these results suggest that both $CD4^+$ Th1 and $CD8^+$ type 1 cytotoxic T cells are crucial effector cells in CHS responses to dinitrofluorobenzene and oxazolone in C57BL/6 mice. *The Journal of Immunology,* **2000, 165: 6783–6790.**

Contact hypersensitivity (CHS)[3] is a T cell-mediated cutaneous immune/inflammatory reaction to haptens (1). The epidermal Langerhans cell (LC), a member of the family of professional APCs, plays a pivotal role in CHS. Depending upon the allergen, LCs can either bind the hapten to MHC molecules on their surface directly or process the allergen internally into a complete Ag (2). LCs then migrate via the afferent lymphatic vessels into skin-draining regional lymph nodes (LNs) to present the haptenated peptide to naive T cells. As a result, T cells become activated and polarized toward a type 1 pattern (afferent phase) (3–5). Upon challenging the skin with the same hapten, the haptenated protein is presented by LCs and/or other APCs to recruited hapten-specific T cells, which are induced to produce the type 1 cytokines IFN-γ and IL-2, thereby initiating the cutaneous inflammatory reaction (efferent phase) (4, 6).

Traditionally, it is believed that CHS represents the prototype of delayed-type hypersensitivity, which is mediated by $CD4^+$ T cells and down-regulated by $CD8^+$ T cells. Circumstantial evidence supporting this view has come from a number of studies. First, in nickel-allergic patients, the presence of nickel-specific $CD4^+$ Th1 cells has been demonstrated in the peripheral blood and skin (7–10). Additionally, it has been shown that T cell lines from FITC-sensitized mice possess a $CD4^+$ Th1-like phenotype, producing large amounts of IFN-γ (11). In vivo depletion of $CD4^+$, but not $CD8^+$, T cells significantly inhibited CHS responses to dinitrofluorobenzene (DNFB) (12, 13). In vitro depletion of $CD4^+$, but not $CD8^+$, T cells from LN cells (LNCs) resulted in a loss of CHS transfer (14). In vitro positively selected $CD4^+$ T cells have been shown to be capable of adoptively transferring CHS to naive mice (15). Priming of naive T cells in vitro with haptenated LCs could induce $CD4^+$ hapten-specific T cells that mediate CHS following injection into naive syngeneic mice (16). CD4 knockout (KO) mice mounted a reduced CHS response (17). $CD4^+$ T cells positively selected from hapten-activated LNCs secreted significant amounts of IFN-γ, whereas $CD8^+$ T cells produced only very low or undetectable levels of this cytokine (18).

Contrary to the traditional understanding of CHS, however, some studies suggest that CHS responses are mediated by $CD8^+$ T cells and down-regulated by $CD4^+$ T cells. For example, clones of $CD8^+$ T cells have been isolated from lesional skin of patients with allergy to urushiol (19). Nickel-specific $CD8^+$ T cells have been detected in patients with allergy to nickel, and these cells possess a type 1 cytotoxic T (Tc1) cell phenotype (20). In vivo depletion of $CD8^+$ T cells resulted in reduction of CHS responses to DNFB and dimethylbenzanthracene (13, 21), whereas deletion of $CD4^+$ T cells led to an exaggeration of the CHS response to DNFB (21). MHC class I-deficient mice were unable to mount a CHS response to DNFB, whereas MHC class II-deficient mice

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[3] Abbreviations used in this paper: CHS, contact hypersensitivity; DC, dendritic cell; DNCB, 2,4-dinitrochlorobenzene; DNFB, dinitrofluorobenzene; KO, knockout; LC, Langerhans cell; LN, lymph node; LNC, LN cell; OXZ, oxazolone; Tc, cytotoxic T; WT, wild type.

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developed an exaggerated CHS response (22). The enhanced CHS responses in MHC class II-deficient mice were decreased by treatment with anti-CD8 Ab, or by injection of wild-type (WT) CD4$^+$ T cells (23). MHC class I$^+$/II$^-$ dendritic cells (DCs) induced hapten-specific immune responses in vitro and in vivo (24), whereas MHC class I$^-$/II$^+$ DCs down-regulated the CHS response (25). T cells in the skin-draining LNs of hapten-sensitized mice showed polarized patterns of cytokine production, with IFN-γ-secreting CD8$^+$ T cells and IL-4/IL-10-secreting CD4$^+$ T cells (26).

In the present study, we examined CHS responses in gene KO mice deficient in CD4$^+$ or CD8$^+$ T cells to directly clarify the requirement for the two T cell subsets in CHS. In addition, we depleted CD8$^+$ T cells from CD4 KO mice or CD4$^+$ T cells from CD8 KO mice in vivo using respective Abs, and then examined alterations of the CHS response. To further elucidate the effector role of CD4$^+$ vs CD8$^+$ T cells in CHS, we performed adoptive transfer experiments using LNCs lacking CD4$^+$ T cells or CD8$^+$ T cells, or both T cell subsets. Finally, we determined cytokine production patterns in LNCs of hapten-sensitized mice by ELISA and intracellular cytokine staining. Our data suggest that both CD4$^+$ Th1 and CD8$^+$ Tc1 cells significantly contribute to the full development of CHS responses to DNFB and oxazolone (OXZ) in C57BL/6 mice.

Materials and Methods

Gene KO mice

The CD4 KO and CD8 KO mice were generated by gene disruption, and the mutation was interbred into a C57BL/6 background, as described previously (27, 28). These mutant mice were maintained under a specific pathogen-free environment. C57BL/6 mice were obtained from the Charles River Breeding Laboratories (Saint Constant, Quebec, Canada) and used as a WT control. All mice were used at 8–12 wk of age. Each experimental group consisted of four to six mice. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Reagents and Abs

DNFB and OXZ were purchased from Sigma (St. Louis, MO). Rat anti-mouse Ia, anti-CD4 (clone YTS 191.1.2), anti-CD8 (clone YTS 169.4), anti-CD4/PE, anti-CD4/FITC, anti-CD8/FITC, goat anti-rat IgG/PE, goat anti-rat IgG/FITC, goat anti-rat IgG/biotin, streptavidin/FITC, NLDC-145, and isotype controls were purchased from Cedarlane (Hornby, Ontario, Canada). Rat anti-mouse IFN-γ (XMG1.2), anti-IL-10 (JES5-2A5), rat IgG1 isotype control (R3-34), anti-IFN-γ/PE, anti-IL-10/PE, anti-CD32/CD16 (2.4G2, Fc block), and hamster anti-mouse CD3ϵ (145-2C11) were purchased from PharMingen Canada (Mississauga, Ontario, Canada).

Immunolabeling of epidermal sheets

The density of epidermal LCs was examined by anti-Ia in situ immunolabeling, as previously described (29). Epidermal sheets were obtained from mouse ears using 0.5 M of ammonium thiocyanate, and labeled with anti-Ia Ab in a three-step immunolabeling procedure. The Ia$^+$ cells (LCs) were counted from coded samples using a micrometer grid.

Assay for CHS

Induction of CHS was conducted using the methods described previously (30, 31). The shaved mouse abdomen skin was painted with 25 μl of 0.5% DNFB in acetone/olive oil (4:1) or 150 μl of 3% OXZ in alcohol/acetone (3:1). Five days later, mice were challenged with 10 μl of 0.2% DNFB or 1% OXZ on each side of the right ear. As a control, the left ear was painted with an identical amount of vehicle. The ear thickness was measured at 24, 48, and 72 h after challenge. Results were expressed as net ear swelling, which was calculated by subtracting the thickness of the vehicle-treated ear from the thickness of the hapten-challenged ear. The percentage response was calculated according to the following formula: response = (net ear swelling in KO mice/net ear swelling in WT mice) $\times$ 100%.

In vivo Ab-blocking studies

Immediately before DNFB challenge, mice were treated with either anti-IFN-γ Ab (XMG1.2) at a dose of 20 μg or anti-IL-10 Ab (JES5-2A5) at a dose of 40 μg in 40 μl PBS. The neutralizing Abs were injected intradermally into one side of each ear of the mouse using a 30-gauge needle under pentobarbital anesthesia. As a control, an equal volume and concentration of nonimmune rat IgG1 (R3-34) were injected into the ear of the control groups. It has been shown that up to 40 μg of rat IgG induces no change in ear swelling (32).

In vivo depletion of T cell subpopulations

For depletion of CD4$^+$ T cells from CD8 KO mice, 100 μg of anti-CD4 Ab (YTS 191.1.2) was injected i.p. for 3 consecutive days. For depletion of CD8$^+$ T cells from CD4 KO mice, rat anti-CD8 Ab (YTS 169.4) was employed. This Ab treatment has been shown to eliminate >95% of the targeted T cell subpopulation (21, 26). The mice were sensitized with DNFB the day following the last injection, and then challenged 5 days later, as described above.

Preparation of LNCs

Skin-draining LNs (i.e., axillary and inguinal LNs) were collected from sensitized mice (5 days post-DNFB sensitization) and naive mice, and single cell suspensions were prepared. Briefly, LNs were gently disrupted by rubbing between the frosted ends of two microscope slides, and then filtered through a nylon mesh cell strainer. LNCs were washed with HBSS and resuspended in complete RPMI 1640 medium (33).

Isolation of migratory hapten-modified LCs

The migratory hapten-modified LCs were isolated from LNCs using metrizamide gradient, as described by Bigby et al. (34). Briefly, WT mice were painted with 0.5% DNFB, and 24 h later LNCs were obtained. LNC suspensions (5 ml) at 5 $\times$ 10^6 cells/ml were layered onto 2 ml of 14.5% metrizamide and gradient centrifuged at 1200 rpm for 15 min. Cells at the interface were collected. Consistent with previous reports, microscopic examination demonstrated that 50–80% of this fraction was Ia$^+$/DEC-205$^+$ DCs (34, 26).

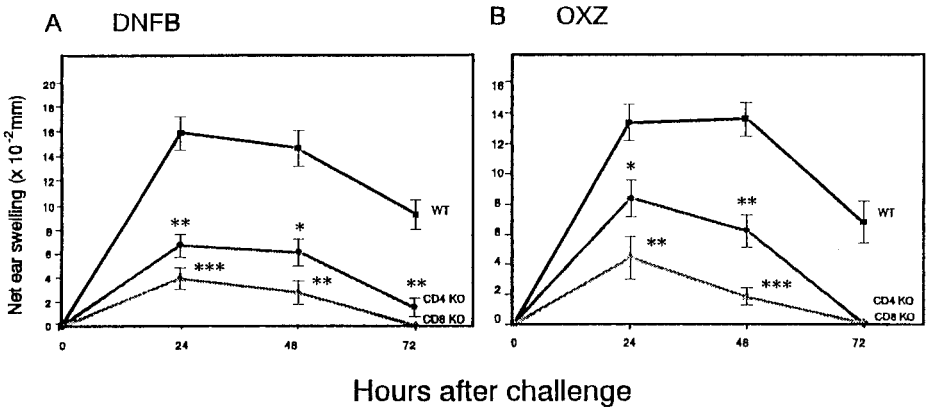

FIGURE 1. Depressed CHS responses in mutant mice lacking either CD4$^+$ or CD8$^+$ T cells. Mice were sensitized with DNFB (*A*) or OXZ (*B*) on the shaved abdomen, and 5 days later challenged on the ear. Ear thickness was measured at various time points. The CHS responses were significantly decreased in CD4 KO mice and CD8 KO mice compared with WT mice (*, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$). The depression of CHS was more significant in CD8 KO mice than in CD4 KO mice ($p < 0.05$).

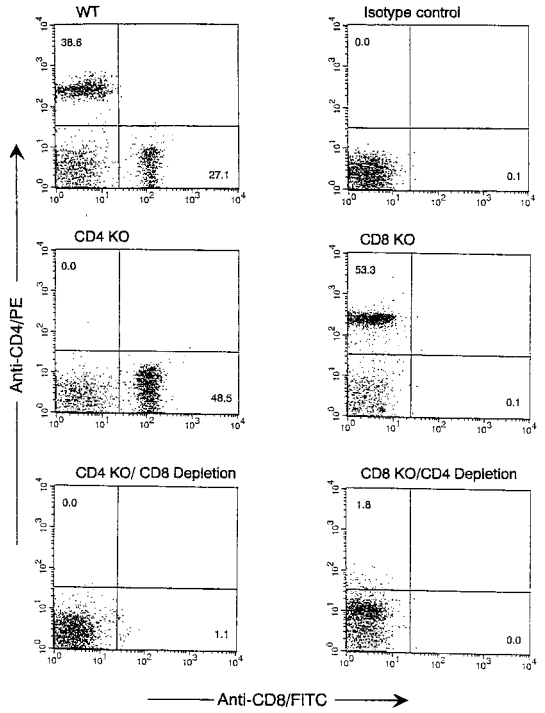

FIGURE 2. FACS analysis on LNCs from Ab-treated mice. CD4 KO mice and CD8 KO mice were injected i.p. with anti-CD8 or anti-CD4 Ab for 3 consecutive days, respectively, and the axillary and inguinal LNs were collected. LNCs were double labeled with anti-CD4/PE and anti-CD8/FITC, and subjected to FACS analysis. The Ab treatment led to elimination of >95% of the targeted T cell subsets.

Preparation of CD4$^+$ T cell and CD8$^+$ T cell double-deficient LNCs

To prepare CD4$^+$ T cell and CD8$^+$ T cell double-deficient LNCs, either CD4$^+$ T cells were depleted in vitro from the CD8 KO LNCs or CD8$^+$ T cells were depleted from the CD4 KO LNCs. The CD8 KO and CD4 KO LNCs were suspended at 2.5×10^7 cells/ml with 25 μg/ml of anti-CD4 or anti-CD8 Ab, respectively, for 60 min on ice. After washing, the cells were resuspended at 2.5×10^7 cells/ml in a 1/9 dilution of Low-Tox-M rabbit complement (Cedarlane) in HBSS/10% FCS and incubated at 37°C for 60 min. These cells were then washed extensively before use in culture or adoptive transfer.

FACS analysis of LNCs

Anti-CD4/PE vs anti-CD8/FITC double staining was performed in LNCs. Briefly, LNCs were preincubated with anti-CD32/CD16 for 5 min, and then incubated with anti-CD4/PE for 30 min on ice. After washing, cells were incubated with anti-CD8/FITC for 30 min. The cells were subjected to FACS analysis. To confirm the efficiency of in vivo depletion of CD8$^+$ T cells in CD4 KO mice, LNCs were first incubated with rat anti-mouse CD8, then reacted with goat anti-rat IgG/FITC, and finally labeled with anti-CD4/PE. To confirm the efficiency of in vivo depletion of CD4$^+$ T cells in CD8 KO mice, LNCs were first incubated with rat anti-mouse CD4 and then reacted with goat anti-rat IgG/PE, and finally labeled with anti-CD8/FITC.

Adoptive transfer of CHS immune response

Four groups of LNCs were prepared from donor mice (5 days post-DNFB sensitization): CD4$^+$ T cell deficient, CD8$^+$ T cell deficient, CD4$^+$ T cell and CD8$^+$ T cell double deficient, and WT LNCs. Naive WT mice were used as recipient mice. LNC suspensions (5×10^7 cells/200 μl HBSS) were injected i.v. into each recipient mouse via the tail vein. The recipient mice were challenged 1 h later by epicutaneous application of DNFB on the ear, and ear swelling was evaluated 24 h later.

Quantitation of in vitro cytokine production

LNCs obtained from sensitized mice (5 days post-DNFB sensitization) and naive mice were stimulated with either plate-bound anti-CD3 or hapten-modified LCs. For anti-CD3 stimulation, 96-well U-bottom plates were precoated with hamster anti-mouse CD3ϵ (25 μg/ml), or hamster IgG isotype control for 90 min at 37°C. LNCs were seeded at 5×10^5 cells/200 μl/well, and incubated at 37°C for 48 h. For stimulation with LCs, 5×10^5 LNCs were cocultured with 10^4 LCs in 200 μl/well for 48 h (26). Next, culture supernatants were collected, and the concentrations of IFN-γ, IL-4, and IL-10 proteins were quantitated by a sandwich ELISA using ELISA kits (Genzyme, Cambridge, MA) (35). Absorbance was read at 450 nm. Each supernatant was analyzed in duplicate, and each experiment was repeated three times.

Intracellular cytokine staining

The frequency of IFN-γ- or IL-10-producing cells in the LNs of DNFB-sensitized or naive C57BL/6 mice was determined using intracellular cytokine staining with Cytofix/Cytoperm Plus kits (PharMingen) (36, 37). LNCs were cultured in 96-well U-bottom plates precoated with anti-CD3ϵ for 24 h, and 2 μM monensin was added for the final 5 h. As a control, LNCs were cultured in media alone. The cells were harvested, preincubated with anti-CD32/CD16, and then stained for the cell surface Ag by incubation with anti-CD4/FITC or anti-CD8/FITC for 30 min on ice. After

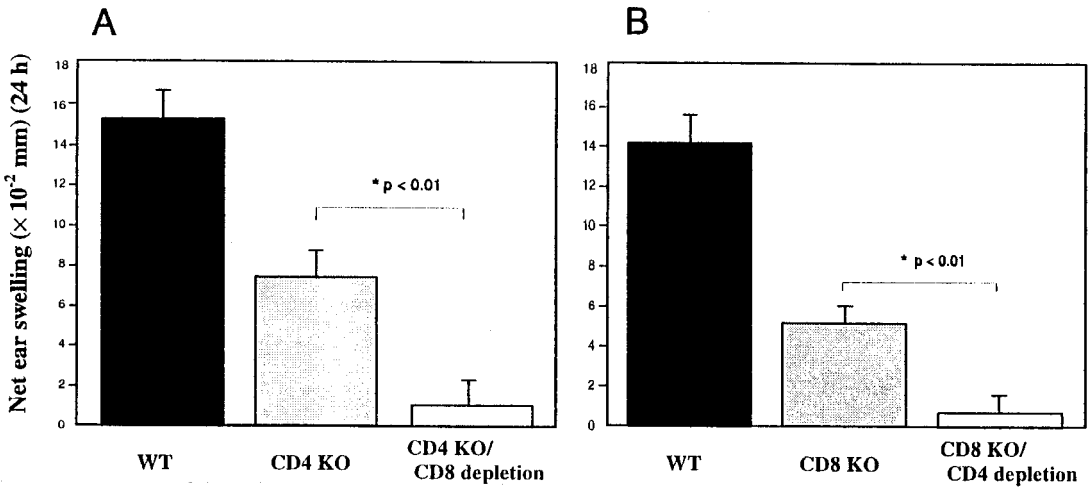

FIGURE 3. In vivo depletion of either CD8$^+$ T cells from CD4 KO mice or CD4$^+$ T cells from CD8 KO mice virtually abolishes CHS responses. CD4 KO (*A*) and CD8 KO (*B*) mice were treated with anti-CD8 or CD4 Ab on 3 consecutive days, respectively, and then sensitized with DNFB. Five days later, the mice were challenged and ear thickness was examined 24 h later. Little or no ear-swelling response was detected in CD8$^+$ T cell-depleted CD4 KO mice and CD4$^+$ T cell-depleted CD8 KO mice.

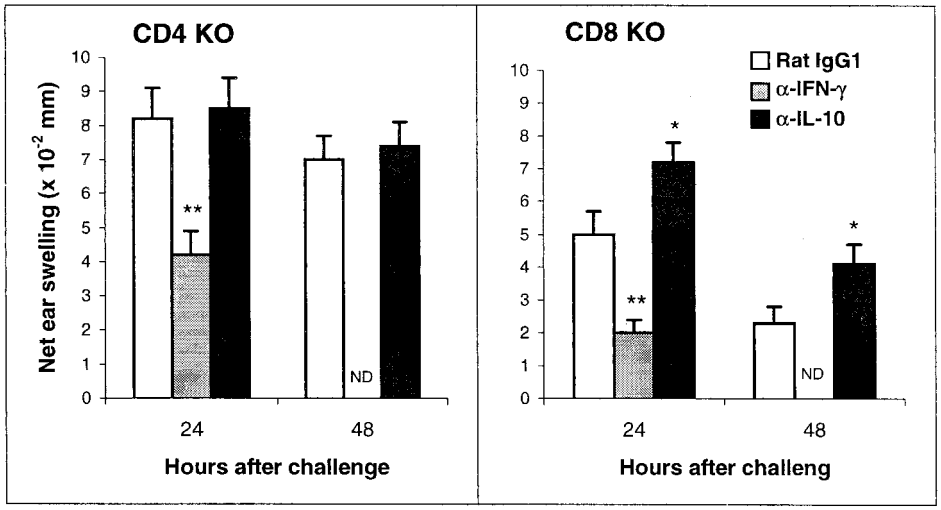

FIGURE 4. In vivo anti-IFN-γ and anti-IL-10 Ab-blocking studies. Immediately before DNFB challenge, CD4 KO and CD8 KO mice were injected intradermally with 20 μg of anti-IFN-γ Ab or 40 μg of anti-IL-10 Ab into the ear. As a control, an equal volume and concentration of rat IgG1 were injected. Anti-IFN-γ Ab suppressed ear swelling in CD4 and CD8 KO mice, whereas anti-IL-10 Ab enhanced CHS in CD8, but not in CD4, KO mice (*, $p < 0.05$; **, $p < 0.01$). ND, Not detectable.

washing, cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, and subsequently stained for intracellular cytokines by incubation with anti-IFN-γ/PE or anti-IL-10/PE, according to the instructions of the manufacturer. As a control, the fixed/permeabilized cells were preincubated with unconjugated anti-IFN-γ or anti-IL-10 before staining. The percentage of cytokine-positive cells was determined by FACScan flow cytometry.

Statistical analysis

A minimum of three experiments was performed for each assay. All data are expressed as the mean and SEM. The statistical significance of differences between the means was determined by applying a two-tailed Student's *t* test. A difference was considered statistically significant with $p < 0.05$.

Results

CHS responses to DNFB and OXZ are depressed in CD4 KO mice and CD8 KO mice

To determine the requirement for the two T cell subpopulations in CHS responses, we performed CHS assays using gene KO mice lacking CD4$^+$ or CD8$^+$ T cells. Before performing the CHS assay, we examined the density of LCs in the epidermis, and demonstrated that normal numbers of LCs were present in CD4 KO and CD8 KO mice (data not shown). Mice were sensitized with DNFB or OXZ, and challenged 5 days later. Ear-swelling responses to DNFB and OXZ were significantly depressed in CD4 KO mice and CD8 KO mice, as compared with WT mice (Fig. 1). The depression of CHS response was more significant in CD8 KO mice than CD4 KO mice ($p < 0.05$). Moreover, the duration of the CHS response was shortened in CD4 KO and CD8 KO mice. Ear-swelling responses returned to baseline in CD4 KO and CD8 KO mice 72 h after challenge, whereas an obvious ear swelling remained in WT mice at this time.

In vivo depletion of either CD8$^+$ T cells from CD4 KO mice or CD4$^+$ T cells from CD8 KO mice virtually abolishes CHS responses

The demonstration that CHS response was depressed, but not completely abrogated in the absence of CD4$^+$ or CD8$^+$ T cells alone raised the possibility that either T cell subset could function as effector cells for CHS. To further address this issue, we in vivo depleted either CD8$^+$ T cells from CD4 KO mice or CD4$^+$ T cells from CD8 KO mice using respective Abs, and then examined CHS responses. FACS analysis on LNCs confirmed that treatment with Ab for 3 consecutive days resulted in elimination of >95% of the targeted T cell subsets (Fig. 2). The resulting CD4$^+$ T cell and

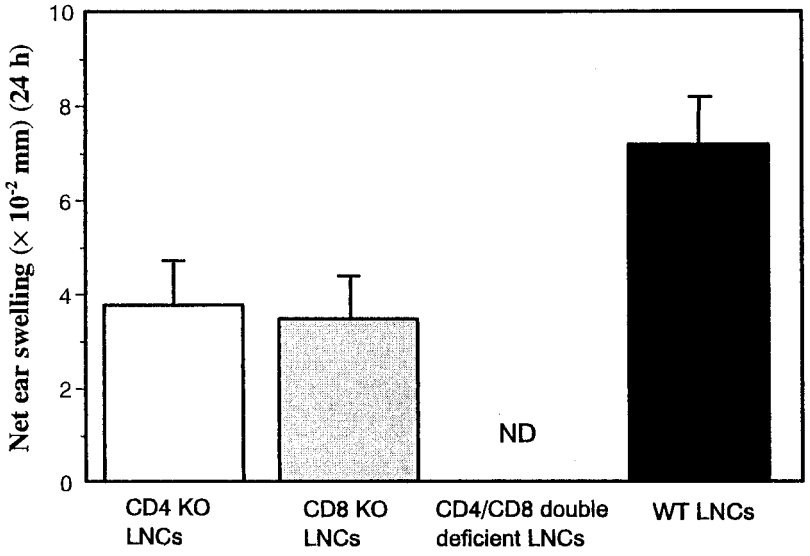

FIGURE 5. Capacity for transferring CHS is impaired in LNCs deficient in either CD4$^+$ or CD8$^+$ T cells. Four groups of donor LNCs were separately injected into recipient naive mice, and then DNFB was painted onto the mouse ear. CD4$^+$ T cell-deficient LNCs and CD8$^+$ T cell-deficient LNCs induced lower CHS responses compared with WT LNCs ($p < 0.01$). Transfer of CD4$^+$ T cell and CD8$^+$ T cell double-deficient LNCs did not induce a CHS response in naive mice. ND, Not detectable.

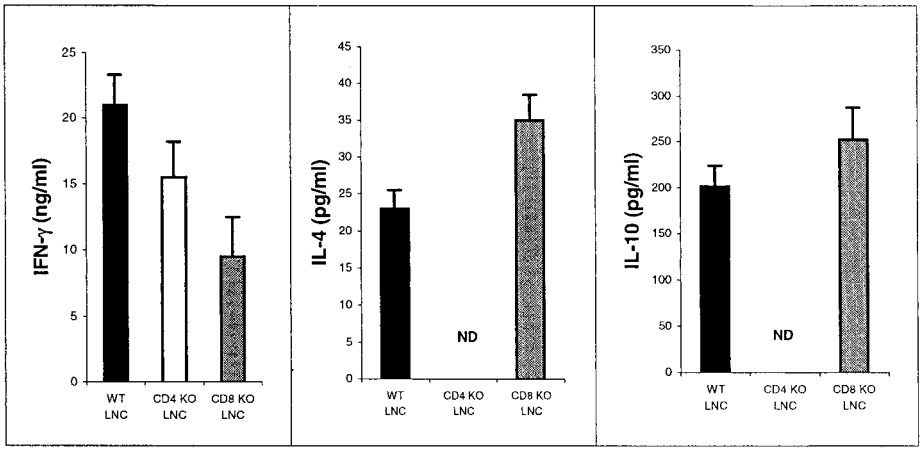

FIGURE 6. Cytokine production of LNCs stimulated by immobilized anti-CD3ε. LNCs from DNFB-sensitized mice were stimulated with plate-bound anti-CD3ε for 48 h, and culture supernatants were subjected to ELISA analysis. Both $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs produced significant amounts of IFN-γ, even though the levels were lower compared with WT LNCs. IL-4 and IL-10 were detected from $CD8^+$ T cell-deficient LNCs as well as WT LNCs, whereas neither of them was detectable from $CD4^+$ T cell-deficient LNCs. ND, Not detectable.

$CD8^+$ T cell double-deficient mice were virtually unable to mount hapten-specific ear-swelling responses (Fig. 3).

Anti-IFN-γ Ab suppresses CHS in CD4 KO and CD8 KO mice, whereas anti-IL-10 Ab enhances CHS in CD8, but not in CD4, KO mice

Before performing Ab-blocking studies in CD4 KO and CD8 KO mice, dose titration tests were performed in WT mice. We found that CHS responses were significantly suppressed by anti-IFN-γ Ab, and enhanced by anti-IL-10 Ab in WT mice in a dose-dependent manner. Maximal effects were obtained when 20 μg of anti-IFN-γ or 40 μg of anti-IL-10 Ab was injected (data not shown). When CD4 KO or CD8 KO mice were injected with 20 μg of anti-IFN-γ Ab, CHS responses were reduced by ~50% at 24 h, and abrogated completely at 48 h in both CD4 KO and CD8 KO mice. On the other hand, injection of 40 μg of anti-IL-10 Ab resulted in a significant enhancement of CHS in CD8, but not in CD4, KO mice (Fig. 4).

LNCs from either CD8 or CD4 KO mice demonstrate a decreased capacity for transferring CHS

To further elucidate the effector role of $CD4^+$ vs $CD8^+$ T cells in CHS, we performed adoptive transfer experiments. The donor cells consisted of four groups of DNFB-primed LNCs: $CD4^+$ T cell deficient, $CD8^+$ T cell deficient, $CD4^+$ T cell and $CD8^+$ T cell double deficient, and WT LNCs. These LNCs were separately injected into recipient naive C57BL/6 mice and then DNFB was painted onto the ear. As shown in Fig. 5, WT, $CD4^+$ T cell-deficient and $CD8^+$ T cell-deficient LNCs were all able to induce a CHS response. However, the capacity of transferring CHS was significantly lower for $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs. Interestingly, transfer of $CD4^+$ T cell and $CD8^+$ T cell double-deficient LNCs did not induce CHS responses in naive mice.

Both $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs produce significant amounts of IFN-γ, whereas only $CD8^+$, but not $CD4^+$, T cell-deficient LNCs secrete IL-4 and IL-10

To determine cytokine profiles in skin-draining LNs of CD4 KO and CD8 KO mice, DNFB-primed LNCs were stimulated with immobilized anti-CD3ε or hapten-modified LCs. As shown in Fig. 6, significant amounts of IFN-γ were detected from anti-CD3-stimulated $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs, implicating that both $CD8^+$ and $CD4^+$ T cells were able to produce the type 1 cytokine. In contrast, IL-4 and IL-10 were only detected from $CD8^+$, but not from $CD4^+$, T cell-deficient LNCs, suggesting that type 2 cytokines were exclusively produced by $CD4^+$ Th2 cells. Significant amounts of IFN-γ or IL-4/IL-10 were not detected from the naive LNCs, or the DNFB-primed LNCs stimulated with control hamster IgG. Similarly, when hapten-modified LCs were used as Ag-specific stimuli, both CD4 KO LNCs and CD8 KO LNCs produced IFN-γ, whereas CD8 KO, but not CD4 KO, LNCs produced IL-4 and IL-10 (Fig. 7). However, the amounts

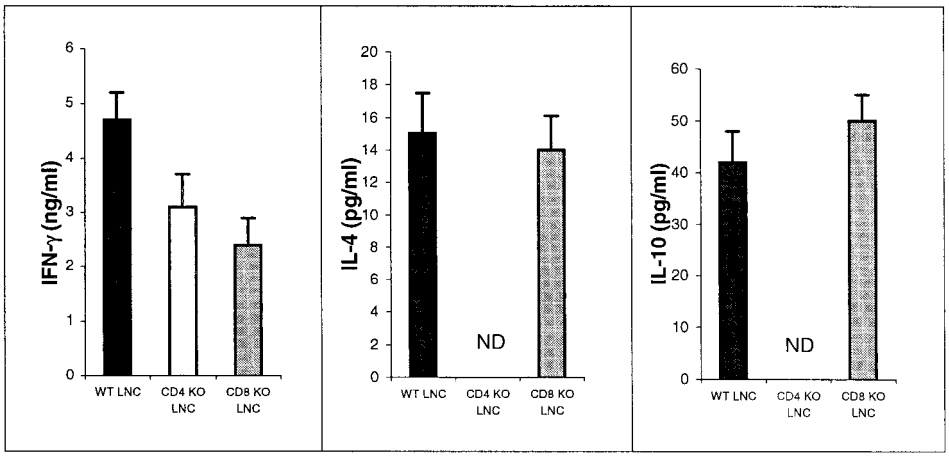

FIGURE 7. Cytokine production of LNCs stimulated with hapten-modified LCs. LNCs from DNFB-sensitized mice were cocultured with hapten-modified LCs for 48 h. Culture supernatants were subjected to ELISA analysis. Following LC stimulation, IFN-γ was detected from $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs, whereas IL-4 and IL-10 were only detectable from $CD8^+$, but not from $CD4^+$, T cell-deficient LNCs.

Table I. *Frequency of intracellular IFN-γ- or IL-10-positive cells in skin-draining LNs*[a]

	CD4$^+$ T Cells		CD8$^+$ T Cells	
	IFN-γ$^+$	IL-10$^+$	IFN-γ$^+$	IL-10$^+$
Media alone				
Naive LNCs	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)
Primed LNCs	0.0 (0.01)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)
Anti-CD3 treatment				
Naive LNCs	0.0 (0.01)	0.0 (0.01)	0.2 (0.02)	0.0 (0.00)
Primed LNCs	8.7 (0.74)	6.8 (0.76)	18.5 (2.16)	0.0 (0.01)

[a] Naive or DNFB-primed LNCs from C57BL/6 mice were cultured in the presence of anti-CD3 and monensin, or in media alone. The cells were surface labeled with anti-CD4/FITC or anti-CD8/FITC and subjected to intracellular cytokine staining with anti-IFN-γ/PE or anti-IL-10/PE. The percentage of positive cells was determined by double-color FACS analysis. Results are derived from three separate experiments and expressed as percentage of cytokine-positive cells in the CD4$^+$ or CD8$^+$ population (mean ± SEM).

of cytokines induced by LCs were considerably lower compared with those induced by immobilized anti-CD3.

Intracellular IFN-γ-positive cells consist of CD4$^+$ Th1 and CD8$^+$ Tc1 cells, whereas IL-10-positive cells are exclusively CD4$^+$ Th2 cells

To confirm the cellular source of cytokines and determine the frequency of cytokine-secreting cells in LNCs, we performed intracellular cytokine staining. After 24 h of culturing LNCs in media alone, significant numbers of cytokine-positive cells were not detected from naive or DNFB-primed LNCs. However, following stimulation of DNFB-primed LNCs with immobilized anti-CD3ε, significant numbers of CD4$^+$ and CD8$^+$ T cells were shown to be IFN-γ positive. In addition, a significant number of IL-10-positive cells were detected from DNFB-primed CD4$^+$, but not CD8$^+$, T cells (Table I). In control experiments, when LNCs were preincubated with unconjugated anti-IFN-γ or anti-IL-10 Ab, no positively labeled cells were detected. As shown in Fig. 8, in a representative experiment, 9.4% of CD4$^+$ T cells (or 3.2% of total LNCs) and 21.4% of CD8$^+$ T cells (or 4.1% of total LNCs) were IFN-γ positive, and 8.2% of CD4$^+$ T cells (or 2.9% of total LNCs) were IL-10 positive. However, significant amounts of IL-10-positive cells were not detected from the CD8$^+$ T cells.

Discussion

The response of T cells to Ag involves the participation of a number of distinct receptor-ligand engagements. The major players in the recognition of complexes of MHC molecules and peptide Ags are the TCR and the T cell coreceptors. CD4 and CD8 molecules are two key coreceptors on T lymphocytes that stabilize and increase the avidity of interaction between the TCR and peptide-MHC determinants on APCs. CD4$^+$ and CD8$^+$ T cell subpopulations make up the vast majority of T cells. The CD4 molecule has demonstrated affinity to MHC class II molecules, hence CD4$^+$ T cells recognize peptide Ags in the Ag-binding pocket of MHC class II molecules. Conversely, CD8$^+$ T cells recognize antigenic peptides in the peptide-binding pockets of MHC class I molecules (38, 39). Therefore, CD4$^+$ T cells and CD8$^+$ T cells have distinct functions. CD4$^+$ T cells are Th cells that play a role in the activation and/or proliferation of B lymphocytes, CTL, and macrophages. CD8$^+$ T cells are usually CTL, which respond to antigenic challenges by lysis of target cells.

Over the last two decades, a large number of studies have focused on the respective role of CD4$^+$ vs CD8$^+$ T cells in CHS; however, conflicting results have been obtained (Table II). The discrepancy may be due to the great diversity in CHS responses. CHS in mice is genetically controlled and differs considerably among strains having different alleles at the *H-2* loci. The response is profoundly modified by non-MHC genes as well. In addition to mouse genetic background, CHS responses vary depending on the hapten (40). Moreover, different types of allergens may even result in qualitatively different immune responses characteristic of selective Th1 and Th2 activation, respectively. Contact allergens such as DNFB, OXZ, and 2,4-dinitrochlorobenzene (DNCB) preferentially induce Th1-predominant responses, whereas respiratory allergens such as trimellitic anhydride induce Th2-predominant responses (41–43). Exceptionally, the contact allergen FITC may also induce a Th2 response (43).

In the present study, we used gene-targeted KO mice deficient in either CD4$^+$ T cells or CD8$^+$ T cells to determine the requirement for, and relative role of, the two T cell subsets. We confirmed that CD4 KO mice had significantly decreased CHS responses to DNFB and OXZ. Moreover, we demonstrated that CHS responses were depressed in CD8 KO mice to an even greater extent. There are two potential reasons for the greater decrease in CHS response in CD8 KO mice: 1) less IFN-γ production and 2) production of the down-regulatory Th2 cytokines IL-4 and IL-10 in these mutant mice. Nevertheless, our data suggest that both CD4$^+$ T cells and CD8$^+$ T cells play an effector role in CHS responses. Our adoptive transfer experiments using LNCs from CD4 KO and CD8 KO

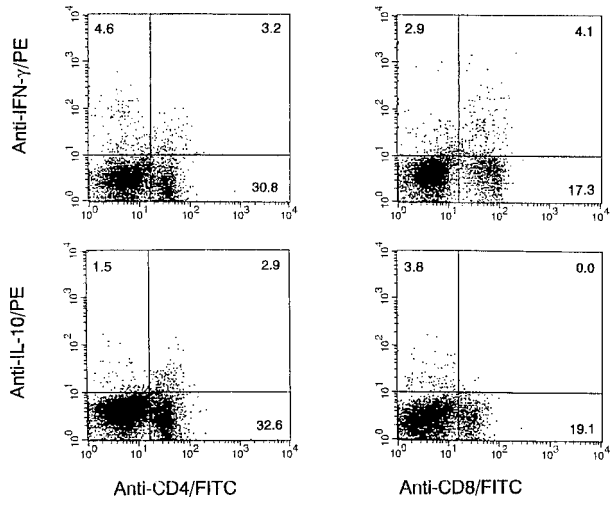

FIGURE 8. Intracellular cytokine staining in DNFB-primed LNCs. LNCs from DNFB-sensitized WT mice were stimulated with plate-bound anti-CD3ε for 24 h, and subjected to intracellular cytokine staining, as described in *Materials and Methods*. Significant numbers of CD4$^+$ T cells and CD8$^+$ T cells were intracellular IFN-γ positive. A significant number of CD4$^+$ T cells were IL-10 positive, whereas virtually no IL-10-positive cells were detected from the CD8$^+$ T cell subpopulation.

Table II. *Contrary results supporting two opposing notions about the effector cells of CHS*

	$CD4^+$ T Cells Mediate CHS	$CD8^+$ T Cells Mediate CHS
Nickel-specific T cells in nickel-allergic patients	$CD4^+$ T cells (7-10)	$CD8^+$ T cells (20)
In vivo depletion of $CD4^+$ T cells	Decreased CHS (12)	Enhanced CHS (21)
In vivo depletion of $CD8^+$ T cells	Normal CHS (12)	Inhibited CHS (21)
In vitro depletion of $CD4^+$ T cells	Decreased transfer (14, 15)	Enhanced transfer (21)
In vitro depletion of $CD8^+$ T cells	Normal transfer (14, 15)	Decreased transfer (21)
MHC class I-deficient mice	Normal CHS (14)	Abolished CHS (22)
Polarization in hapten-primed LNCs	IFN-γ-producing $CD4^+$ Th1 cells (11, 18)	IFN-γ-producing $CD8^+$ Tc1 T cells (26)

mice support this notion. The experimental results revealed that both the $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs had a decreased capacity of transferring CHS. Additionally, $CD4^+$ T cell and $CD8^+$ T cell double-deficient LNCs were unable to transfer CHS at all.

Although CHS responses were depressed in mutant mice deficient in $CD4^+$ or $CD8^+$ T cells, the ear-swelling response was not completely lost. Therefore, we hypothesized that $CD8^+$ T cells may mediate the remaining CHS response seen in CD4 KO mice, and $CD4^+$ T cells may be responsible for the CHS responses seen in CD8 KO mice. To test this hypothesis, we performed in vivo $CD4^+$ or $CD8^+$ T cell depletion studies. Abs were used to remove $CD4^+$ T cells from $CD8^+$ KO mice or $CD8^+$ T cells from CD4 KO mice. We found that the CHS response was virtually abolished in these $CD4^+$ T cell and $CD8^+$ T cell double-deficient mice. These results suggest that both $CD4^+$ T cells and $CD8^+$ T cells contribute to the full development of CHS responses.

Previous studies using gene KO mice and neutralizing Abs have demonstrated that cytokines play important effector and regulatory roles in CHS responses. The type 1 cytokine IFN-γ promotes CHS, whereas the type 2 cytokines, IL-10 and to a lesser extent IL-4, downregulate CHS responses (44–47). Our in vivo Ab-blocking studies in CD4 KO and CD8 KO mice support this notion. Administration of anti-IFN-γ Ab before challenge resulted in a reduction of CHS in both CD4 KO and CD8 KO mice, whereas anti-IL-10 Ab significantly enhanced ear swelling in CD8, but not in CD4, KO mice.

It has been demonstrated that type 1 cytokines can be produced by $CD4^+$ Th1 cells as well as $CD8^+$ Tc1 cells, whereas type 2 cytokines can be derived from Th2 and Tc2 cells (48, 49). Determination of the cellular sources of IFN-γ and IL-4/IL-10 in the hapten-primed LNCs was very helpful for determining the relative role of T cell subpopulations in CHS responses. Several groups have examined the cytokine profiles in LNCs of mice following hapten sensitization. Xu et al. (26) demonstrated that in vivo depletion of $CD8^+$ T cells in mice resulted in a decrease of IFN-γ production by DNFB-primed LNCs, whereas depletion of $CD4^+$ T cells led to an increasing IFN-γ secretion. This suggests that IFN-γ is mainly produced by $CD8^+$ Tc1 cells. In contrast, Moussavi et al. (18) found that $CD4^+$ T cells positively selected from DNCB-primed mouse LNCs produced IFN-γ, whereas $CD8^+$ T cells secreted only very low or undetectable levels of IFN-γ. This discrepancy may be accounted for by the differences in the experimental protocol. For example, in one case DNFB was used as the contact sensitizer, while in the other case DNCB was used. Moreover, Ab treatments are not able to completely remove all $CD4^+$ or $CD8^+$ T cells, and may even trigger other responses from T cells.

In the present study, we examined cytokine production patterns utilizing LNCs from CD4 KO and CD8 KO mice, which are completely deficient in $CD4^+$ or $CD8^+$ T cells. LNCs were stimulated with either immobilized anti-CD3 or hapten-modified LCs. ELISA analysis revealed that both $CD4^+$ T cell-deficient LNCs and $CD8^+$ T cell-deficient LNCs were capable of producing IFN-γ, and that $CD8^+$, but not $CD4^+$, T cell-deficient LNCs could produce IL-4 and IL-10. These results suggest that IFN-γ is derived from both $CD4^+$ T cells and $CD8^+$ T cells, while IL-4 and IL-10 are produced only by $CD4^+$ T cells. Although the observation that type 2 cytokines are exclusively derived from $CD4^+$ T cells in LNCs is consistent with the data of Xu et al. (26), the results concerning the main cellular source of type 1 cytokine are different. One explanation for this discrepancy is that different sensitization protocols were employed. Instead of two daily DNFB paintings, we used a single painting for DNFB sensitization (50, 51).

To confirm the observations from the ELISA study, we performed intracellular cytokine staining on the DNFB-primed LNCs from WT mice. Since the quantities of cytokines induced by hapten-modified LCs were considerably lower compared with those induced by immobilized anti-CD3, we used anti-CD3-stimulated LNCs for intracellular cytokine staining. Consistently, Xu et al. (26) demonstrated that anti-TCR or anti-CD3 Ab was able to stimulate LNCs to produce higher levels of cytokines than LCs. Furthermore, Matyszak et al. (52) recently demonstrated that the amount of cytokines produced during primary Ag-specific immune responses was very small, which could be enhanced by restimulation with anti-CD3. They also demonstrated (by intracellular staining) that none or very little IFN-γ-, IL-4-, or IL-10-positive T cells was detected from DC-stimulated Ag-specific $CD4^+$ T cells in the absence of anti-CD3. However, when restimulated with anti-CD3, significant amounts of cytokine-positive T cells were detected. Our intracellular cytokine staining confirmed that immobilized anti-CD3 was able to induce significant amount of cytokine-producing cells in LNCs. We demonstrated that IFN-γ-positive cells in the skin-draining LNs of sensitized mice consisted of both $CD4^+$ (Th1) T cells and $CD8^+$ (Tc1) T cells, whereas IL-10-positive cells were exclusively $CD4^+$ (Th2) T cells.

Collectively, the present study provides evidence that both $CD4^+$ T cells and $CD8^+$ T cells are required for the full development of CHS. $CD4^+$ Th1 and $CD8^+$ Tc1 cells are both effector cells, while $CD4^+$ Th2 cells are negative regulatory cells for the CHS responses to DNFB and OXZ in C57BL/6 mice.

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