T Cell Populations Primed by Hapten Sensitization in Contact Sensitivity Are Distinguished by Polarized Patterns of Cytokine Production: Interferon \( \gamma \)-producing (Tc1) Effector CD8\(^+\) T Cells and Interleukin (II) 4/II-10–producing (Th2) Negative Regulatory CD4\(^+\) T Cells

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Summary
Contact hypersensitivity (CHS) is a T cell–mediated response to hapten sensitization of the epidermis. The roles of CD4\(^+\) and CD8\(^+\) T cells in CHS have remained unclear, however, as studies to define either subset as the T cells mediating CHS have provided conflicting results. The goal of this study was to correlate the in vivo function of CD4\(^+\) and CD8\(^+\) T cells in CHS with the cytokines produced by each T cell population. Antibody-mediated depletion of CD4\(^+\) T cells before sensitization of BALB/c mice with 2,4-dinitrofluorobenzene (DNFB) or oxazolone (Ox) resulted in increased and prolonged CHS responses, indicating CD4\(^+\) T cells as negative regulators of the response. Depletion of CD8\(^+\) T cells resulted in low or abrogated responses, indicating CD8\(^+\) T cells as the effector cells in CHS. Sensitization with DNFB or Ox induced lymph node cell populations of CD8\(^+\) T cells producing interferon (IFN)-\( \gamma \) and no interleukin (II) 4 or II-10, and CD4\(^+\) T cells producing II-4 and II-10 and no or little detectable IFN-\( \gamma \). The polarized patterns of cytokine production were stimulated by culture of hapten–primed lymph node cells either on anti–T cell receptor antibody–coated wells or with semipurified Langerhans cells isolated from hapten-sensitized mice. Stimulation of cytokine production during culture of hapten-primed CD4\(^+\) or CD8\(^+\) T cells with Langerhans cells was hapten specific and restricted to class II or class I major histocompatibility complex, respectively. The induction of the CD4\(^+\) and CD8\(^+\) T cells producing the polarized patterns of cytokines was not restricted to BALB/c mice, as cells from Ox sensitized C57Bl/6 and B10.D2 mice produced the same patterns. Collectively, these results expose the induction of two polarized and functionally opposing populations of T cells by hapten sensitization to induce CHS: IFN-\( \gamma \)-producing effector CD8\(^+\) T cells and II-4/II-10–producing CD4\(^+\) T cells that negatively regulate the response.

Abbreviations used in this paper: CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; LC, Langerhans cells; hpl.C, hapten-presenting Langerhans cells; LNC, lymph node cells; Ox, oxazolone.
mune response (8–11). Studies by Gocinski and Tigelaar (12), however, have indicated the ability of immune CD8^+ T cells to mediate CHS and the ability of immune CD4^+ T cells to inhibit the response. Overall, these studies have used differences in the magnitude of CHS responses in the absence or presence of immune CD4^+ and/or CD8^+ T cells to define the cellular components of the response. Although CHS is actually mediated by the cytokines produced by primed T cells, cytokine production by hapten-specific CD4^+ and CD8^+ T cells in CHS has remained largely undefined.

During antigen priming, CD4^+ T cells develop from IL-2-producing precursor (i.e., Th0) cells into one of two distinct phenotypes of cytokine-producing cells. Th1 cells produce IL-2, IFN-\(\gamma\), and TNF-\(\beta\), whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (for review see reference 13). The different patterns in cytokine production reflect the different immune functions performed by these phenotypes of CD4^+ T cells. DTH reactions are mediated by Th1 cells, whereas Th2 cells provide signals required for the generation of humoral responses (11, 13, 14). An important aspect of the polarized production of cytokines is the regulation of Th1 responses by Th2 cytokines and vice versa. The magnitude of DTH reactions is inhibited by Th2 cytokines, particularly IL-4 and IL-10 (15, 16). Similarly, Th2 function and cytokine production are inhibited by the Th1 cytokine IFN-\(\gamma\) (17). Whereas antigen-primed CD8^+ T cells usually produce IFN-\(\gamma\), in vitro studies have indicated that CD8^+, like CD4^+, T cells can be skewed into IFN-\(\gamma\)-producing (Tc1) or IL-4/IL-10–producing (Tc2) cells by the cytokine conditions present during antigen priming (18, 19).

Similar to results observed in DTH, reagents either inhibiting the induction of Th1 cells or neutralizing Th1 cytokines result in decreased CHS responses. Treatment with either Th2 cytokines (i.e., IL-4 or IL-10) or antibodies neutralizing Th1 cytokines (i.e., anti-IFN-\(\gamma\) or anti-TNF-\(\alpha\) antibodies) reduces the magnitude of CHS (6, 20–23). Alternatively, in vivo administration of antibodies neutralizing Th2 cytokines (i.e., anti-IL-4 or anti-IL-10 antibodies) enhances CHS (20, 22). Since these results suggested that CHS may be mediated by Th1 cells and regulated by Th2 cells, we examined the cytokines produced by hapten-primed T cells. During initial experiments using antibody-mediated depletion of CD4^+ or CD8^+ T cells in vivo, we observed results similar to Gocinski and Tigelaar (12), suggesting that the elicitation of CHS responses required immune CD8^+ T cells and that the magnitude of the response was regulated by CD4^+ T cells. When the cytokines produced by hapten-immune CD4^+ and CD8^+ T cells from either dinitrofluorobenzene (DNFB)– or oxazolone (Ox)–sensitized mice were evaluated, the CD8^+ T cells produced IFN-\(\gamma\) and no detectable IL-4 or IL-10, and the CD4^+ T cells produced IL-4 and IL-10 and little or no IFN-\(\gamma\). These results expose the induction of two polarized and functionally opposing populations of T cells by hapten sensitization to induce CHS: IFN-\(\gamma\)–producing Tc1 CD8^+ T cells as the effector cells of CHS and IL-4/IL-10–producing Th2 CD4^+ T cells that negatively regulate the response. The induction of the Th2 regulatory, rather than a Th1 effector, population of CD4^+ T cells demonstrates an important distinction between CHS and classical DTH reactions.

Materials and Methods

Mice. BALB/c and C57Bl/6 mice were obtained through Dr. Clarence Reeder at the National Cancer Institute (Frederick, MD). B10.D2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Adult females 6–10 wk of age were used throughout this study.

Antibodies and Reagents. The following mAbs were obtained from the American Type Culture Collection (Rockville, MD): GK1.5 (anti-mouse CD4), MKD6 (anti-I-A^B), 14.4.4S (anti-I-E^K), HB102 (anti-D^b), and HB159 (anti-K^B). mAbs from the culture supernatant of the IgG-producing hybridomas YTS 169.4.2.1 (anti-mouse CD8 [24]), HS7-597 (anti-TcR CB), and, KJ23a (V\(\beta\)17a [26]), as well as those listed above, were purified by protein G chromatography. Capture and detection of mAbs for cytokine-specific ELISA and recombinant cytokines for standardization of assays were purchased from PharMingen (San Diego, CA).

Sensitization and Elicitation of CHS. Mice were sensitized and challenged to elicit CHS responses to DNFB and Ox as previously described (27). For the induction of CHS to DNFB, groups of three mice were sensitized by two daily paintings (days 0 and +1) with 25 \(\mu\)g of 0.5% DNFB (Sigma Chemical Co., St. Louis, MO) on the shaved abdomen and 5 \(\mu\)l on the footpads. For the induction of CHS to Ox, groups of three to four mice were painted on the shaved abdomen once (day 0) with 50 \(\mu\)l of 3% Ox (Aldrich Chemical Co., Milwaukee, WI) and 5 \(\mu\)l on the footpads. Sensitized and unsensitized control animals were challenged on day +5 by applying 10 \(\mu\)l of 0.2% DNFB or 1% Ox to each side of both ears. Increase in ear swelling (\(\delta\)) was measured in a blinded manner 24 h after challenge with an engineer’s micrometer (Mitsutoyo Precision USA, Inc., Elk Grove Village, IL) and expressed in units of 10^-4 in as previously described (27). The magnitude of ear swelling (see Fig. 1) is given as the mean increase of each group of three individual animals (i.e., six ears) minus the swelling in unsensitized mice challenged with the hapten ± SEM.

Antibody Treatment for Depletion of T Cells. In vitro depletion of CD4^+ or CD8^+ T cells was performed by treatment with specific antibody and complement. Briefly, lymph node cells (LNC) from hapten-sensitized donors were suspended at 50 \(\times\) 10^6/ml with 10 \(\mu\)g/ml of GK1.5 (anti-CD4), YTS 169 (anti-CD8), or control (rat IgG) antibody in RPMI 1640. After 40 min on ice, the cells were washed, resuspended at 10^6/ml in rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). After 45 min at 37°C, the cells were washed extensively before use in culture. In vivo, CD4^+ or CD8^+ T cells were depleted by intraperitoneal injection of 100 \(\mu\)g of YTS 169 (anti-CD8 mAb) or GK1.5 (anti-CD4 mAb) on three consecutive days as described by Cobbold and coworkers (24). This treatment resulted in depletion of \(\approx\)96% of the target T cell population as assessed by flow cytometry analysis (data not shown). Antibody-treated mice were rested 1–3 d before shaving and sensitization with DNFB or Ox.

Isolation of Hapten-presenting Langerhans Cells (hpLC). The isolation of hpLC was performed using the protocol of Bigby and coworkers (4). To isolate DNFB hpLC, mice were painted on two consecutive days with 0.5% DNFB, and LNC were obtained 20–24 h after the final painting. To isolate Ox hpLC, mice were painted once with 3% Ox, and LNC were obtained 20–24 h...
later. The LNC were washed twice in HBSS and resuspended at 6 × 10^6 cells/ml in complete medium (RPMI 1640 supplemented with 5 mM glutamine, 100 U/ml penicillin–streptomycin, and 10% heat-inactivated FCS). The cell suspension (6–8 ml) was layered over 2 ml of 14.5% metrizamide (Sigma Chemical Co.) in PBS and centrifuged at 600 g for 10 min. The interface cells were collected and washed twice with complete medium. Consistent with the results of Bigby and coworkers (4), microscopic and flow cytometry examination of cells from the interface contained 60–80% slg^-class II MHC^+ cells with dendritic cell morphology.

**Cell Culture.** LNC were obtained either from unsensitized or from sensitized mice on day +4 and stimulated to produce cytokines by two different culture methods. For stimulation by culture on antibody-coated wells, 96 U-bottom-well tissue culture plates were precoated with 30 µl/well of anti-TCR (anti-TCR CB, H57-597, or as a negative control, anti-Vβ17a, KJ23a) antibody at 25 µg/ml for 90 min at 37°C. The wells were washed extensively, and 2 × 10^5 naive or hapten-immune LNC were delivered to each well in 200 µl complete medium. After 48 h, culture supernatants were harvested and assayed for cytokine production by ELISA. For stimulation by culture with hpLC, 2 × 10^5 naive or hapten-immune LNC were cocultured with 10^4 hpLC, unless otherwise indicated, in 200 µl/well in 96 U-bottom-well tissue culture plates. Supernatants were harvested and assayed for cytokine production 48 h later. In experiments determining the role of MHC antigens in the activation of hapten-immune T cells to produce cytokines, purified anti-class I MHC and/or anti-class II MHC antibodies were included in the cultures at 50 µg/ml.

**ELISA.** Cytokine-specific sandwich ELISA for determining quantities of IFN-γ, IL-2, IL-4, and IL-10 were performed using capture and detection antibodies from PharMingen, generally following the instructions of the supplier. For each antibody pair, the concentrations of capture and detection antibodies were optimized in initial experiments using recombinant cytokines. Briefly, polyvinyl chloride ELISA plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with antibodies in 0.1 M bicarbonate buffer, pH 8.6, during overnight incubation at 4°C. The plates were washed and blocked with 5% FCS plus 0.05% gelatin in PBS for 2 h at 37°C. After extensive washing, duplicate aliquots of test supernatants were delivered to the wells. All supernatants were tested undiluted and in at least 2–3 dilutions. Each plate also included 9–10 dilutions of the test recombinant cytokine as a positive control and to obtain a standard curve for quantitation. After overnight incubation at 4°C, each plate was washed extensively, and the biotin-labeled detection antibody was added. The plate was incubated for 1 h at 37°C, washed extensively, and streptavidin–alkaline–phosphatase (Fisher Scientific Co., Pittsburgh, PA) added. After a final incubation of 45 min at 37°C, each plate was washed and the assay developed by addition of the substrate, p-nitrophenyl phosphate (Sigma Chemical Co.). Results were read at 405 nm using an automatic ELISA plate reader (Bio-Tek Instruments Inc., Winooski, VT) and mean values obtained. The amount of cytokine in each test supernatant was calculated according to a standard curve derived from the use of serially diluted recombinant cytokines performed in each plate.

**Results**

**Increased and Prolonged CHS Responses in the Absence of CD4^+ T Cells.** To begin to examine the roles of CD4^+ and CD8^+ T cells in CHS, we treated BALB/c mice with antibodies to deplete CD4^+ or CD8^+ T cells and then sensitized the animals for CHS with either DNFB (Fig. 1 A) or Ox (Fig. 1 B). After hapten challenge to the ears, change in ear thickness was determined at 24-h increments for 5 d. Typical of CHS responses in this model, the magnitude of ear swelling in control antibody–treated animals rapidly decreased from the peak observed at 24–48 h after challenge and was at background levels by 72–96 h after challenge. The effect of anti-CD4 antibody treatment observed in each response was virtually identical in that depletion of CD4^+ T cells resulted in a higher response than the control antibody–treated mice. In the absence of CD4^+ T cells, the DNFB response was maintained as a plateau at the peak magnitude for 72 h after challenge. Furthermore, in CD4^+ T cell–depleted animals, the responses to both DNFB and Ox decreased slowly and did not reach background levels until 120–192 h after challenge. Depletion of CD8^+ T cells had the opposite effect as depletion of CD4^+ T cells, resulting in a lower (40% of positive control) ear swelling response to Ox 24 h after challenge and a more striking decrease in the response to DNFB, lowering the response to background levels. The low or absent CHS responses ob-

![Figure 1](image-url)
served in CD8+ T cell–depleted animals were maintained at the low levels for at least 120 h after hapten challenge (data not shown). These results confirm those originally reported by Gocinski and Tigelaar (12) and suggest that CD8+ T cells mediate CHS responses, whereas CD4+ T cells regulate the magnitude of the response.

Polarization of Cytokine Production by Immune CD4+ and CD8+ T Cells after Hapten Sensitization. Since the results in Fig. 1 suggested that CD4+ and CD8+ T cells may have different and possibly opposing functions during CHS, we examined the cytokines produced after in vitro stimulation of CD4+ and CD8+ T cells from DNFB- or Ox-sensitized mice. The first approach taken was to stimulate hapten-immune LNC by culture with anti-TCR antibody–coated wells and test the culture supernatants for cytokine production by ELISA. In preliminary experiments, culture of DNFB- or Ox-immune LNC with either anti-TCR Cβ antibody H57-597 or anti-CD3ε antibody stimulated similar and detectable levels of cytokines in the supernatant 48 h after culture initiation (data not shown). Cytokine production was not stimulated either during culture of immune LNC on control anti-TCR (i.e., anti-β17α) antibody–coated wells or during culture of LNC from unsensitized mice on H57-597 (data not shown). Whereas H57-597 stimulation of Ox-immune cells from control antibody–treated mice resulted in the production of low but detectable levels of IL-2, IL-4, and IFN-γ and higher levels of IL-10, stimulation of Ox-immune LNC from anti-CD4 and anti-CD8 antibody–pretreated animals revealed a polarized pattern of cytokine production (Fig. 2 A). Immune (CD8+) cells from anti-CD4 antibody–treated animals produced higher levels of IFN-γ than immune cells from control antibody–treated mice but nondetectable levels of IL-2, IL-4, and IL-10. In contrast, immune (CD4+) cells from anti-CD8 antibody–treated animals produced higher levels of IL-2, IL-4, and IL-10 than the control and a barely detectable level of

![Figure 2. Anti-TCR antibody–stimulated cytokine production by LNC from hapten-sensitized mice. BALB/c mice were given 100 μg of rat IgG (immune T Cells) or anti-CD4 (Immune CD8+ Cells) or anti-CD8 (Immune CD4+ Cells) mAb on three consecutive days. 2 d later, the animals were sensitized once (day 0) with 3% Ox (A) or twice (days 0 and +1) with 0.5% DNFB (B). On day +4, LNC suspensions from sensitized or unsensitized (Naive T Cells) animals were prepared, and 2 × 10⁶ cells were cultured on anti-TCR antibody (H57-597)-coated wells. Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine. ND, not detectable.]
IFN-γ. As indicated above, stimulation of LNC from unsensitized animals did not result in IL-4, IL-10, and IFN-γ production, although very low amounts of IL-2 were detected in a few experiments.

The distinct patterns of cytokine production by CD4+ and CD8+ T cells were also observed after stimulation of immune LNC from DNFB-sensitized mice pretreated with anti-CD8 or anti-CD4 antibodies. Similar to results observed with Ox-immune cells, DNFB-immune (CD8+) cells from anti-CD4 antibody–treated mice produced higher levels of IFN-γ and no IL-2 or IL-4 after stimulation with the anti-TCR antibody, and (CD4+) cells from mice pretreated with anti-CD8 antibody produced IL-2 and IL-4 but barely detectable amounts of IFN-γ (Fig. 2 B). In contrast to the Ox-immune CD4+ T cells, production of IL-10 by the DNFB-immune cells from anti-CD8 antibody–treated mice was undetectable. In addition, the quantity of IL-4 produced by DNFB-immune CD4+ T cells after H57 stimulation was always considerably lower when compared with the amount produced by Ox-immune CD4+ T cells (e.g., 212 pg/ml for Ox vs. 77 pg/ml for DNFB).

To ensure that the patterns of cytokine production observed were not a trivial effect attributable to the in vivo antibody-mediated depletion of CD4+ and CD8+ T cells, the cytokines produced by immune LNC depleted of CD4+ or CD8+ T cells in vitro was assessed. Aliquots of LNC from Ox-sensitized mice were treated with control, anti-CD4, or anti-CD8 antibody and complement. After washing, the cells were cultured on H57-597–coated wells and the supernatants analyzed by ELISA 48 h later. Immune cells treated with control (rat IgG) antibody and complement produced detectable levels of IL-4, IL-10, and IFN-γ after culture on anti-TCR antibody–coated wells (Fig. 3). As observed in the previous experiment, naive LNC did not produce IL-4 or IL-10 during culture on anti-TCR antibody, although a small amount of IFN-γ was detected. Depletion of CD4+ T cells from the immune cells removed cells producing IL-4 and IL-10, whereas cells producing IFN-γ remained. Depletion of CD8+ T cells from the immune population did not affect cells producing IL-4 and IL-10 but reduced IFN-γ production to background levels. Collectively, the results shown in Figs. 2 and 3 indicated a segregated pattern of cytokine production by hapten-sensitized T cells with immune CD8+ T (Tc1) cells producing IFN-γ and immune CD4+ T (Th2) cells producing IL-2, IL-4, and IL-10.

Hapten-immune T Cell Cytokine Production Stimulated by hpLC. Whereas anti-TCR antibody was shown to stimulate cytokine production by immune CD4+ and CD8+ T cells from hapten-sensitized mice, activation of T cells is normally mediated by cellular presentation of antigen–MHC complexes. The hapten-presenting cells priming T cell responses in CHS are LC, which migrate from the sensitized epidermis to the skin-draining lymph nodes (3–5, 28). To examine hapten-primed CD4+ and CD8+ T cell cytokine production using a more physiological system, we isolated semipurified LC from the lymph nodes of mice 24 h after painting with Ox as described in Materials and Methods and tested the ability of the hpLC to stimulate IL-4 and IFN-γ production by Ox-immune LNC from mice pretreated with anti-CD4 or anti-CD8 antibodies. The amount of IL-4 produced by Ox-immune CD4+ T cells (from CD8-deleted animals) during coculture with Ox hpLC was dependent on the number of LC in the culture (Fig. 4). Detectable levels of IL-4 were observed when as few as 5,000 hpLC were cultured with the immune CD4+ T cells. Similarly, production of IFN-γ by Ox-immune CD8+ T cells was dependent on the number of LC added to the culture with detectable cytokine stimulated by as few as 2,500 LC.

Although the quantities produced were considerably lower than those observed after stimulation by anti-TCR antibody, the hpLC stimulated immune LNC from Ox-sensitized mice pretreated with control antibody to produce IL-2, IL-4, and IFN-γ (Fig. 5 A). LNC from unsensitized mice were not stimulated to produce detectable levels of cytokines during culture with the hpLC. Immune lymph node CD8+ T cells from animals pretreated with anti-CD4 antibody produced increased amounts of IFN-γ (when compared with the cells from the control immune mice) and no IL-2 or IL-4 during coculture with the hpLC. Immune CD4+ T cells from anti-CD8 antibody–treated mice

![Figure 3. Cytokine production by Ox-immune CD4+ and CD8+ T cells. BALB/c mice were sensitized once with 3% Ox, and 4 d later LNC suspensions were prepared and treated first with anti-CD4 (Immune CD8+ Cells) or anti-CD8 (Immune CD4+ Cells) antibody and then with complement. Control (Immune T Cells) cells were treated with complement only. After washing, 2 × 10^5 treated immune or nontreated naive cells were cultured on anti-TCR antibody (H57-597)–coated wells. Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine. ND, not detectable.](image-url)
produced increased amounts of IL-2 or IL-4 and barely detectable amounts of IFN-γ during coculture with the hpLC. In contrast to culture on anti-TCR–coated wells, the production of IL-10 during culture of immune LNC with the hpLC was never observed during the course of these experiments.

When semipurified LC from DNFB-sensitized animals were tested for the ability to stimulate DNFB-immune LNC to produce cytokines, similar results to the Ox response were observed. Culture of immune LNC from anti-CD8 antibody–treated mice produced higher amounts of IL-4 than immune cells from control antibody–treated mice during culture with hpLC, and immune LNC from anti-CD4 antibody–treated mice produced higher amounts of IFN-γ than the nondeleted control immune cells (Fig. 5 B). The quantities of IL-4 and IFN-γ produced by the DNFB-immune LNC during culture with the hpLC were consistently lower than the amounts produced during coculture of Ox-immune LNC and Ox-presenting LC.

The ability of the hpLC to stimulate CD4+ and CD8+ T cells to produce cytokines in a hapten-specific manner was then examined. Whereas immune CD4+ and CD8+ T cells from Ox-sensitized mice produced the segregated pattern of cytokines during culture with hpLC isolated from Ox-sensitized mice, cytokine production was undetectable during culture of the Ox-immune cells with hpLC isolated from DNFB-sensitized mice (Table 1). Similarly, immune CD4+ and CD8+ T cells from DNFB-sensitized mice produced detectable amounts of IL-4 and IFN-γ, respectively, during culture with hpLC from DNFB-sensitized mice but not during culture with hpLC isolated from Ox-sensitized mice.

The ability of anti–class I MHC and anti–class II MHC antibodies to inhibit the hpLC-stimulated production of cytokines by hapten-immune CD4+ and CD8+ T cells was examined. Immune LNC from Ox-sensitized mice pre-treated with either anti-CD4 or anti-CD8 antibody were cultured with hpLC from Ox-sensitized mice in the presence of antibodies specific for class I MHC (Kd, HB159, or Dd, HB102) or class II MHC (I-Ak, MKD6, or I-Ekd, 14,4.4S). Supernatants were harvested 48 h after culture initiation and the quantities of IFN-γ and IL-4 were determined (Table 2). Consistent with the observations in this report, immune T cells from anti-CD4 antibody–treated animals were stimulated to produce IFN-γ and no detectable IL-4 during culture with hpLC, whereas immune T cells from anti-CD8 antibody–treated animals were stimulated to produce IL-4 and barely detectable IFN-γ during the culture. The presence of anti–class II MHC antibodies in the culture had virtually no effect on the production of IFN-γ by the immune CD8+ T cells. IFN-γ production was inhibited in the presence of either anti-Kd (55% inhibition) or anti-Dd (40% inhibition) antibodies. This inhibition was never complete, however, even when the anti-Kd and anti-Dd antibodies were used in combination. Production of IL-4 by the immune CD4+ T cells (i.e., from anti-CD8 antibody–treated animals) during culture with the hpLC was completely blocked in the presence of anti-I-E antibody but was only partially blocked (44% inhibition) in the presence of the I-A antibody. Although the hpLC-stimulated production of IL-4 by immune CD4+ T cells was not inhibited by the anti-Kd antibody, addition of the anti-Dd antibody to the culture consistently resulted in lower (30–43% inhibition) quantities of IL-4 produced by the CD4+ T cells. Reasons for the low level of inhibition by this antibody are unclear at this time. These results indicate that the immune T cells from hapten-sensitized mice have conventional MHC restriction patterns for activation in that the CD4+ Th2 cell production of IL-4 is restricted to class II MHC, and the CD8+ Tc1 cell production of IFN-γ is, at least partially, restricted to class I MHC.

**Induction of Polarized Responses in B10.D2 and C57Bl/6 Mice.** In several model systems, BALB/c mice have a tendency to mount a Th2 rather than a Th1 response to test antigens (29, 30). To determine the induction of Tc1 CD8+ T cells and Th2 CD4+ T cells after hapten sensitization in other strains of mice, we examined the cytokine-producing phenotypes of T cells in C57Bl/6 and B10.D2 mice. Groups of mice were treated with control, anti-CD4, or anti-CD8 antibodies and sensitized with Ox. As observed with cells from BALB/c mice, LNC from Ox-sensitized C57Bl/6 (Fig. 6 A) and B10.D2 (Fig. 6 B) mice pretreated with control antibody produced readily detectable amounts

![Figure 4](image-url)

**Figure 4.** hpLC stimulate immune T cells to produce cytokines. BALB/c mice were given 100 μg of anti-CD4 (Immune CD4+ Cells) or anti-CD8 (Immune CD8+ Cells) mAb on three consecutive days. 2 d later, the animals were sensitized once with 3% Ox. 4 d later, LNC suspensions were prepared, and 2 × 10^5 cells were cultured with the indicated number of semipurified hpLC from Ox-sensitized mice. The hpLC were isolated from LNC suspensions 24 h after Ox painting by metrizamide gradient centrifugation. Culture supernatants were collected 48 h later and analyzed by ELISA for quantity of IFN-γ and IL-4.
Naive T Cells

Immune T Cells

Immune CD8⁺ Cells

Immune CD4⁺ Cells

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Concentration (pg/ml)

0 10 20 30 40 50 60 70

IL-2

ND

0 10 20 30 40 50

Concentration (pg/ml)

b

Naive T Cells

Immune T Cells

Immune CD8⁺ Cells

Immune CD4⁺ Cells

Concentration (pg/ml)

0 10 20 30 40 50

IL-4

ND

0 2 4 6 8 10

Concentration (ng/ml)

b

Naive T Cells

Immune T Cells

Immune CD8⁺ Cells

Immune CD4⁺ Cells

Concentration (pg/ml)

0 10 20 30 40 50

IFN-γ

ND

Figure 5. hpLC stimulate immune CD4⁺ and CD8⁺ T cells to produce the polarized pattern of cytokines. BALB/c mice were given 100 μg of rat IgG (Immune T Cells) or anti-CD4 (Immune CD8⁺ Cells) or anti-CD8 (Immune CD4⁺ Cells) mAb on three consecutive days. 2 d later, the animals were sensitized once (day 0) with 3% Ox (A) or twice (days 0 and +1) with 0.5% DNFB (B). On day +4, LNC suspensions from sensitized or unsensitized (Naive T Cells) animals were prepared, and 2 × 10⁶ cells were cultured with 10⁴ hpLC isolated from lymph nodes 24 h after painting with Ox (A) or DNFB (B). Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine. ND, not detectable.

Table 1. Stimulation of Primed T Cell Cytokine Production by hpLC Is Hapten Specific

| mAb pretreatment | Hapten sensitization | IFN-γ | IL-4 | IFN-γ | IL-4 | IFN-γ | IL-4 |
|------------------|----------------------|-------|------|-------|------|-------|------|
| None             | None                 | ND    | ND   | ND    | ND   | ND    | ND   |
| None             | Ox                   | 5.6   | 22   | ND    | ND   | 180   | 225  |
| Anti-CD4         | Ox                   | 7.6   | ND   | ND    | ND   | 387   | ND   |
| Anti-CD8         | Ox                   | ND    | 81   | ND    | 6    | 703   | ND   |
| None             | DNFB                 | ND    | ND   | 2.6   | 16   | 62    | 38   |
| Anti-CD4         | DNFB                 | ND    | ND   | 8.6   | ND   | 200   | ND   |
| Anti-CD8         | DNFB                 | ND    | ND   | 33    | ND   | 30    | 77   |

BALB/c mice were given 100 μg of rat IgG (None) or anti-CD4 or anti-CD8 mAb on three consecutive days. 2 d later, the animals were sensitized once (day 0) with 3% Ox or twice (day 0 and +1) with 0.5% DNFB. On day 14, LNC suspensions from sensitized or unsensitized animals were prepared, and 2 × 10⁶ cells were cultured either with 10⁴ hpLC isolated from lymph nodes 24 h after painting with Ox or DNFB or on anti-TCR antibody (H57-597)–coated wells. Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine. The unit for IFN-γ is nanograms per milliliter and for IL4 is picograms per milliliter. ND, not detectable.
balt has been reported (32, 33). The isolation of urushiol-reactive, T cells in CHS have remained unclear. Whereas in vivo shown to be dependent on the activity of hapten-specific T cells, an undifferentiated population (e.g., Th0) producing I1-2 in addition to Th2 cytokines or the presence of two distinct populations of hapten-immune CD4+ T cells, the characterization of CD4+ T cell clones from the lesions of patients with allergic contact dermatitis to nickel and cobalt has been reported (32, 33).

The results of this study clearly demonstrate the induction of both CD4+ and CD8+ T cells during hapten sensitization and distinguish the role of each population during the elicitation of the CHS response. Antibody-mediated depletion of CD4+ T cells before hapten sensitization resulted in prolonged CHS responses of increased magnitude, whereas depletion of CD8+ T cells resulted in complete abrogation of the response to DNFB and a 40–80% reduction in the response to Ox (Fig. 1). These results confirm experiments and results reported by Gociński and Tigelaar (12). Furthermore, these investigators demonstrated that depletion of CD4+ T cells from immune LNC enhanced the passive transfer of the CHS response to DNFB, whereas depletion of CD8+ T cells abrogated the transfer of CHS (12). These results are contrary to studies of other investigators indicating that CD4+, and not CD8+, T cells mediate CHS (8, 9). This discrepancy may be due to inefficient antibody-mediated depletion of CD8+ T cells in the latter studies. In support of the proposed roles of CD4+ and CD8+ T cells in CHS are recent results from this laboratory examining the CD4+ and CD8+ T cell–dependent expression of proinflammatory cytokine (i.e., chemokine) genes during the elicitation of CHS in which we observed that expression of an IFN-γ–induced chemokine gene (IP-10) was mediated by immune CD8+ T cells (Abe, M., T. Kondo, H. Xu, and R.L. Fairchild, manuscript submitted for publication). Moreover, the expression of IP-10 during CHS was amplified in the absence of CD4+ T cells and was inhibited by the activity of CD8+ T cells. These results suggested that IFN-γ production in CHS was mediated by CD8+ T cells and that CD4+ T cells may inhibit IFN-γ production or its downstream effects.

The effector role of CD8+ T cells and the regulatory role of CD4+ T cells in CHS are supported by the polarized pattern of cytokines produced by each T cell population. Sensitization with either DNFB or Ox induced both hapten–specific CD8+ T cells producing IFN-γ and no I1-4 or II-10, and hapten–specific CD4+ T cells producing II-4 and II-10 and no or little IFN-γ. Anti-TCP.. antibody activity of Ox- and DNFB-immune CD4+ T cells also stimulated production of the Th2 cytokine IL-5 (data not shown). In addition to the Th2 cytokines, stimulation of the CD4+ T cells resulted in II-2 production. This could indicate either the ability of the hapten–immune CD4+ T cells to produce II-2 in addition to Th2 cytokines or the presence of two distinct populations of hapten-immune CD4+ T cells, an undifferentiated population (e.g., Th0) producing II-2 and a Th2 population producing II-4, II-5, and II-10. Experiments to distinguish these possibilities are currently in progress. The role of IFN-γ as one of the major cytokine mediators of CHS and the ability of II-4 and II-10 to inhibit CHS responses have been well established (6, 7, 20–23). The direct role of II-4 in regulating CHS has been further suggested in studies by Gautam and co-workers (20) in which anti-II-4 antibody given at the time of hapten challenge, but not sensitization, resulted in responses of increased magnitude. In contrast, we have observed that anti-II-4 antibody given during hapten sensitization also resulted in increased CHS responses (Xu, H., unpublished observations). Since the induction of Th2 responses has been shown to be dependent on II-4 (34–36), it is tempting to speculate that neutralization of II-4 during sensitization may inhibit the development of the CD4+ Th2 regulatory population during sensitization and subsequently result in increased CHS responses.

**Table 2. Inhibition of Hapten-primed T Cell Cytokine Production by Anti-MHC Class I or Class II Antibodies**

| mAb pretreatment | mAb in the culture | IFN-γ (ng/ml) | II-4 (pg/ml) |
|------------------|--------------------|--------------|--------------|
| Anti-CD4         | None               | 11.6 ND      | ND           |
| Anti-K d         | 5.2                | ND           | ND           |
| Anti-I-A d       | 10.1               | ND           | ND           |
| Anti-D d         | 7.0                | ND           | ND           |
| Anti-I-E k/d     | 10.0               | ND           | ND           |
| Anti-K d + anti-D d | 6.7              | ND           | ND           |
| Anti-CD8         | None               | 1.8 120      | ND           |
| Anti-K d         | 1.0                | 120          | ND           |
| Anti-I-A d       | 2.1                | 68           | ND           |
| Anti-D d         | 1.9                | 81           | ND           |
| Anti-I-E k/d     | ND                 | ND           | ND           |
| Anti-I-A d + anti-I-E k/d | ND         | ND           | ND           |

BALB/c mice were given 100 μg of anti-CD4 or anti-CD8 mAb on three consecutive days. 2 d later, the animals were sensitized once, (day 0) with 3% Ox. On day +4, LNC suspensions from sensitized animals were prepared, and 2 × 10^6 cells were cultured with 10^6 hplc isolated from lymph nodes 24 h after painting with Ox in the presence of the indicated anti-MHC antibodies at 50 μl/ml. Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine. ND, not detectable.

Tc1 CD8+ and II-4/II-10–producing Th2 CD4+ T cells in CHS is not restricted to BALB/c mice.

**Discussion**

Although the elicitation of CHS responses has been shown to be dependent on the activity of hapten–specific T cells, the requirement for and the roles of CD4+ and CD8+ T cells in CHS have remained unclear. Whereas in vivo and in vitro antibody depletion studies from several laboratories have indicated CD4+ T cells as the effector T cells in CHS, others have observed the ability of CD8+ T cells to mediate CHS (8, 9, 12). The isolation of urushiol–reactive, IFN-γ–producing CD8+ T cells from the skin lesions of patients sensitized to poison ivy is supportive of the proposed effector role of CD8+ T cells in CHS (31). In support of the role of CD4+ T cells as the effectors of CHS, the characterization of CD4+ T cell clones from the lesions of patients with allergic contact dermatitis to nickel and cobalt has been reported (32, 33).

The results of this study clearly demonstrate the induction of both CD4+ and CD8+ T cells during hapten sensitization and distinguish the role of each population during the elicitation of the CHS response. Antibody-mediated depletion of CD4+ T cells before hapten sensitization resulted in prolonged CHS responses of increased magnitude, whereas depletion of CD8+ T cells resulted in complete
Figure 6. Hapten sensitization induces polarized patterns of cytokine production by CD4+ and CD8+ T cells in C57BL/6 and B10.BR mice. C57BL/6 (A) or B10.BR (B) mice were given 100 μg of rat IgG (Immune T Cells) or anti-CD4 (Immune CD8+ Cells) or anti-CD8 (Immune CD4+ Cells) mAb on three consecutive days. 2 d later, the animals were sensitized once with 3% Ox. 4 d later, LNC suspensions from sensitized or unsensitized (Naive T Cells) animals were prepared, and 2 × 10^6 cells were cultured on anti-TCR antibody (1-157-597)-coated wells. Supernatants were collected 48 h later and analyzed by ELISA for quantity of the indicated cytokine.

The polarized cytokine patterns produced by hapten-primed CD4+ and CD8+ T cells were revealed through stimulation with either anti-TCR antibody or hpLC. The quantities of IFN-γ and IL-4 produced by hapten-immune CD8+ and CD4+ T cells, respectively, were higher in the absence of the reciprocal T cell population, suggesting a degree of cross-regulation. Whereas readily detectable amounts of IFN-γ were stimulated during culture of Ox-immune CD8+ T cells on anti-TCR antibody, detectable IL-4 was not produced during culture of the cells with hpLC. It is possible that production of IL-10 by hapten-sensitized CD4+ T cells requires a strong TCR-mediated activation signal that is provided by the anti-TCR antibody but not by the hpLC. This differential production of IL-10 by the hapten-primed CD4+ T cells could indicate that CD4+ T cells produce IL-4 but not IL-10 during the CHS response. Several observations support the hypothesis that IL-10-mediated inhibition of CHS may be the result of innate or non-T cell immune mechanisms. First, studies by Enk and Katz (37) have indicated that hapten sensitization stimulates keratinocytes to produce IL-10. Second, Ferguson and coworkers (22) have reported that the highest amount of IL-10 protein in the hapten-challenged tissue of immune mice was detected at 10–14 h after challenge, which is several hours before the peak magnitude of the CHS response. Furthermore, the quantity of IL-10 in the hapten-challenged ear tissue of naive and sensitized mice was the same (Ferguson, T., personal communication). In light of these results, CD4+ T cell regulation of CHS, as demonstrated in this and other reports (12), may be mediated through the production of IL-4. This is difficult to reconcile with recent results from Berg and coworkers (38) indicating normal CHS responses to Ox in mice with targeted disruption of the IL-4 gene but enhanced responses in mice with targeted disruption of the IL-10 gene.

A critical factor influencing CD4+ T cell development to the Th1 or Th2 phenotype is the cytokine environment during T cell encounter with APC. The generation of Th2 CD4+ T cells requires IL-4 in the priming environment (34–36), whereas APC production of IL-12 skews CD4+ T cells to the Th1 phenotype (39, 40). The hapten-presenting cells priming T cell responses in CHS are LC, which migrate from the sensitized epidermis to the skin-draining lymph nodes (3–5). The induction of the Th2, rather than a Th1, CD4+ T cell population in CHS suggests that the hapten-presenting LC do not produce IL-12 during priming of the CD4+ T cells. This prediction is consistent with the observed production of IL-10 by keratinocytes after hapten sensitization and the ability of IL-10–treated LC to stimulate the activation of Th2 CD4+ T cells but not Th1 cells (37,
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