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 (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)-γ and no interleukin (Il) 4 or Il-10, and CD4$^+$ T cells producing Il-4 and Il-10 and no or little detectable IFN-γ. 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-γ–producing effector CD8$^+$ T cells and Il-4/Il-10–producing CD4$^+$ T cells that negatively regulate the response.

Contact hypersensitivity (CHS)[1] is a T cell–mediated immune response in the epidermis to a reactive hapten covalently coupled to cell surface proteins (1, 2). During the afferent or sensitization phase, the hapten-specific T cells mediating the CHS reaction are primed by Langerhans cells (LC), which migrate from the sensitized area of the epidermis to the skin-draining lymph nodes and present hapten–MHC complexes to the peripheral T cell repertoire (3–5). Subsequent contact or challenge with the hapten results in cutaneous infiltration of the primed T cells and their activation to produce TNF-α and IFN-γ, the cytokine mediators of the CHS reaction (6, 7). T cell production of proinflammatory cytokines is followed by the recruitment and infiltration of other inflammatory cells to the challenge site and the characteristic edema, peaking at 24–48 h after challenge. Although CHS has been used in many laboratories as a model to examine T cell responses to simple haptens presented in association with MHC determinants, studies to define CD4$^+$ or CD8$^+$ T cells as the cellular mediators in CHS have yielded conflicting results. Results from several laboratories have indicated that the CHS response is mediated by CD4$^+$ T cells and have led to the perception that CHS is equivalent to the classical delayed-type hypersensitivity (DTH) reaction, which is primarily an IFN-γ–producing CD4$^+$ T cell–mediated im-

[1] *Abbreviations used in this paper:* CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; LC, Langerhans cells; hpLC, hapten-presenting Langerhans cells; LNC, lymph node cells; Ox, oxazolone.

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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-γ, and TNF-β, 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-γ (17). Whereas antigen-primed $CD8^+$ T cells usually produce IFN-γ, in vitro studies have indicated that $CD8^+$, like $CD4^+$, T cells can be skewed into IFN-γ–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-γ or anti–TNF-α 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 examined, the $CD8^+$ T cells produced IFN-γ and no detectable Il-4 or Il-10, and the $CD4^+$ T cells produced Il-4 and Il-10 and little or no IFN-γ. These results expose the induction of two polarized and functionally opposing populations of T cells by hapten sensitization to induce CHS: IFN-γ–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^d), 14.4.4S (anti–I-$E^{k/d}$), HB 102 (anti-D^d); and HB159 (anti-K^d). mAbs from the culture supernatant of the IgG-producing hybridomas YTS 169.4.2.1 (anti–mouse CD8 [24]), H57-597 (anti-TcR Cβ [25]), and, KJ23a (Vβ17a [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 μl of 0.5% DNFB (Sigma Chemical Co., St. Louis, MO) on the shaved abdomen and 5 μl 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 μl of 3% Ox (Aldrich Chemical Co., Milwaukee, WI) and 5 μl on the footpads. Sensitized and unsensitized control animals were challenged on day +5 by applying 10 μl of 0.2% DNFB or 1% Ox to each side of both ears. Increase in ear swelling (δ) was measured in a blinded manner 24 h after challenge with an engineer's micrometer (Mitutoyo 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 50×10^6/ml with 10 μg/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 10^8/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 μg 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 ≥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% sIg^-/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 Cβ, 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-

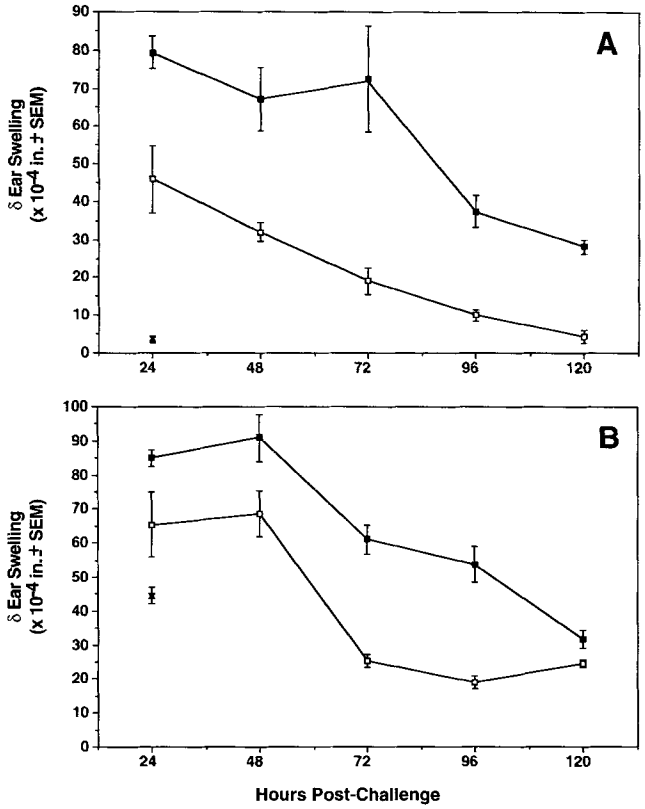

Figure 1. Contact sensitivity responses in the absence of $CD4^+$ or $CD8^+$ T cells. BALB/c mice were given 100 μg of rat IgG (□) or anti-CD4 (■) or anti-CD8 (X) mAb on three consecutive days. 2 d later, the animals were sensitized twice (days 0 and +1) with 0.5% DNFB (*A*) or once (day 0) with 3% Ox (*B*). On day +5, each ear was challenged on both sides with 0.2% DNFB (*A*) or 1% Ox (*B*). The thickness of hapten-challenged ears was measured at 24-h increments and expressed as change (δ) in ear swelling $\times 10^{-4}$ in ± SEM when subtracted from swelling in unsensitized mice challenged with hapten.

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 andTigelaar (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-Vβ17a) 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

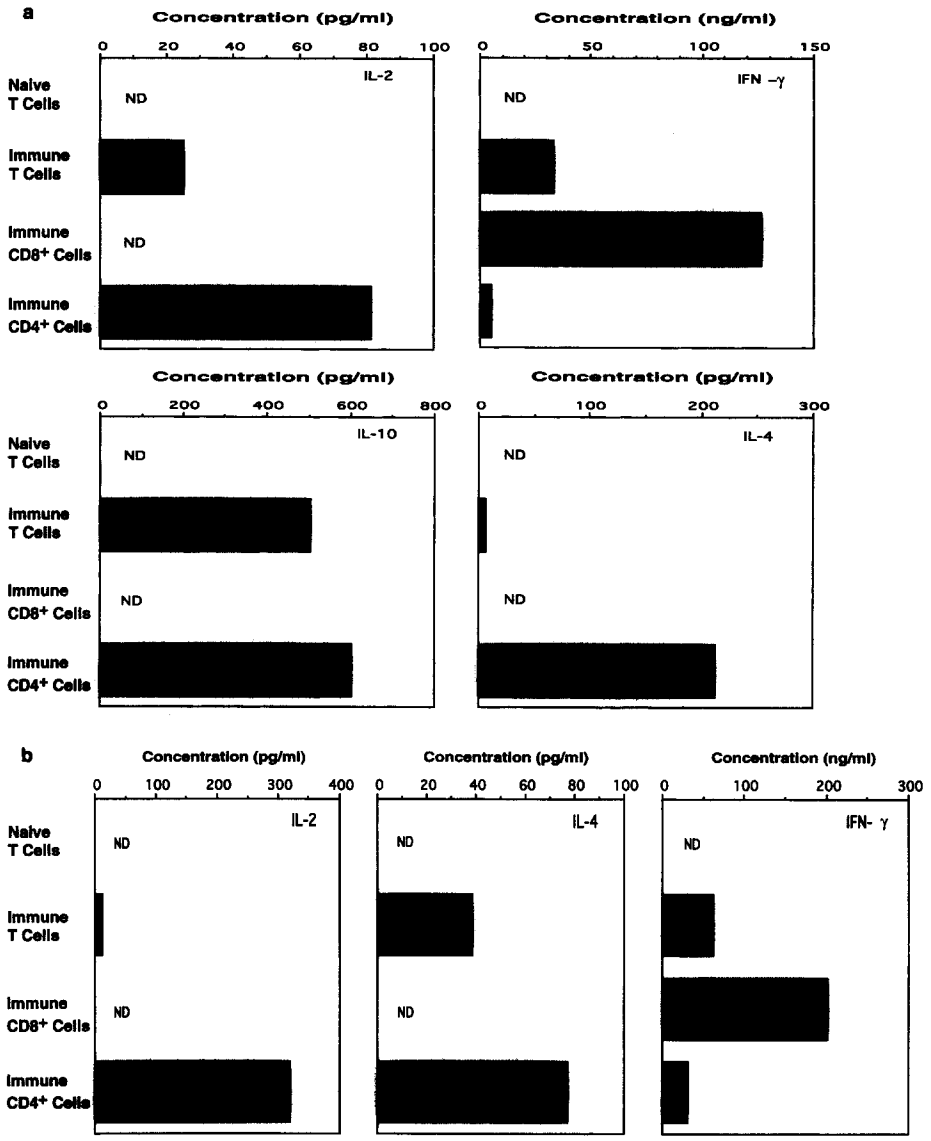

Figure 2. Anti-TCR antibody–stimulated cytokine production by LNC from hapten-sensitized mice. BALB/c mice were given 100 μg of rat IgG (*Immune T Cells*) or anti-CD4 (*Immune $CD8^+$ Cells*) or anti-CD8 (*Immune $CD4^+$ Cells*) mAb on three consecutive days. 2 d later, the animals were sensitized once (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 2×10^5 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 high 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-senstized 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

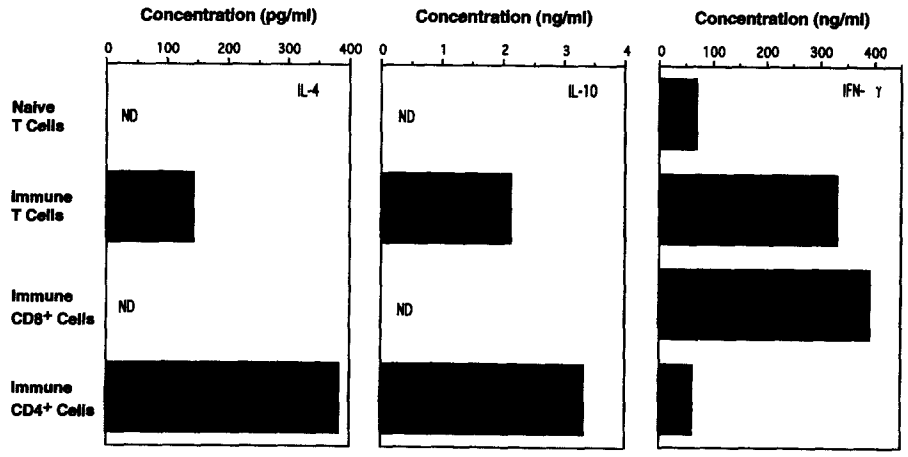

Figure 3. Cytokine production by Ox-immune $CD4^+$ and $CD8^+$ T cells. BALB/c mice were sensitized once with 3% Ox, and 4 d later LNC suspensions were prepared and treated first with anti-CD4 (*Immune $CD8^+$ Cells*) or anti-CD8 (*Immune $CD4^+$ Cells*) antibody and then with complement. Control (*Immune 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.

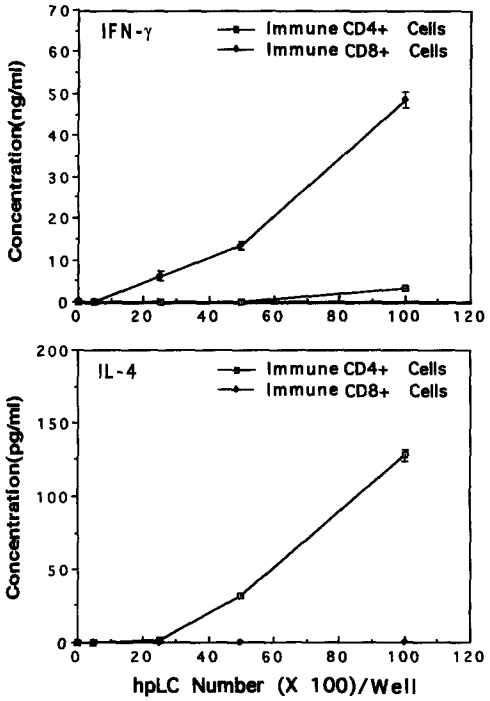

Figure 4. hpLC stimulate immune T cells to produce cytokines. BALB/c mice were given 100 μg of anti-CD4 (*Immune CD8⁺ Cells*) or anti-CD8 (*Immune CD4⁺ Cells*) mAb on three consecutive days. 2 d later, the animals were sensitized once with 3% Ox. 4 d later, LNC suspensions were prepared, and 2×10^5 cells 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.

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 pretreated 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 (K^d, HB159, or D^d, HB102) or class II MHC ($I\text{-}A^d$, MKD6, or $I\text{-}E^{k/d}$, 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-K^d (55% inhibition) or anti-D^d (40% inhibition) antibodies. This inhibition was never complete, however, even when the anti-K^d and anti-D^d 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-K^d antibody, addition of the anti-D^d 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

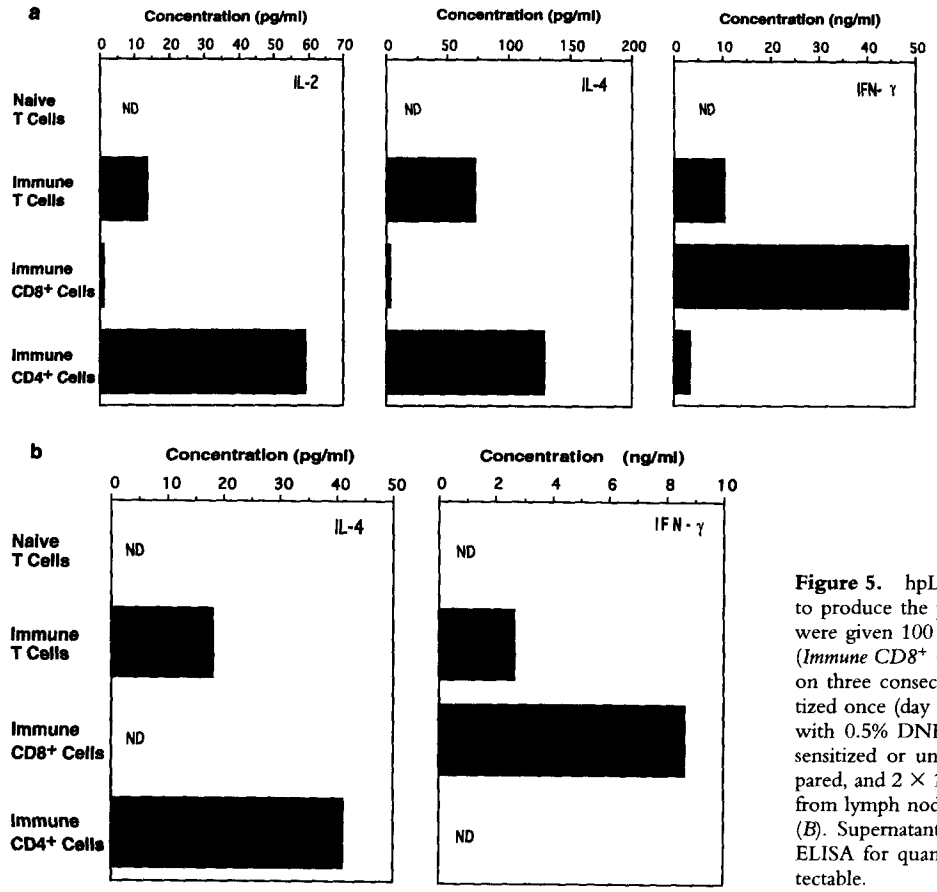

Figure 5. hpLC stimulate immune $CD4^+$ and $CD8^+$ T cells to produce the polarized pattern of cytokines. BALB/c mice were given 100 μg of rat IgG (*Immune T Cells*) or anti-CD4 (*Immune $CD8^+$ Cells*) or anti-CD8 (*Immune $CD4^+$ Cells*) mAb on three consecutive days. 2 d later, the animals were sensitized 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 2×10^5 cells were cultured with 10^4 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.

of Il-4, Il-10, and IFN-γ after stimulation with H57. In each of the tested strains of mice, deletion of $CD4^+$ T cells before sensitization resulted in increased IFN-γ production but no detectable Il-4 or Il-10 during the culture with anti-TCR antibody. Deletion of $CD8^+$ T cells resulted in increased Il-4 and Il-10 production while virtually eliminating the production of IFN-γ. These results indicate that the induction of polarized populations of IFN-γ producing

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

		Cytokine production induced by					
		Ox–hpLC		DNFB–hpLC		H57	
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	ND	6	703
None	DNFB	ND	ND	2.6	16	62	38
Anti-CD4	DNFB	ND	ND	8.6	ND	200	ND
Anti-CD8	DNFB	ND	ND	ND	33	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 2×10^5 cells were cultured either with 10^4 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.

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	Il-4
		(ng/ml)	*(pg/ml)*
Anti-CD4	None	11.6	ND
	Anti-K^d	5.2	ND
	Anti-I-A^d	10.1	ND
	Anti-D^d	7.0	ND
	Anti-I-$E^{k/d}$	10.0	ND
	Anti-K^d + anti-D^d	6.7	ND
Anti-CD8	None	1.8	120
	Anti-K^d	1.0	120
	Anti-I-A^d	2.1	68
	Anti-D^d	1.9	81
	Anti-I-$E^{k/d}$	ND	ND
	Anti-I-A^d + anti-I-$E^{k/d}$	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 2×10^5 cells were cultured with 10^4 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 Il-4/Il-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 dermitis 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 Gocinski 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 discrepency 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 $CD4^+$ 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 Il-4 or Il-10, and hapten-specific $CD4^+$ T cells producing Il-4 and Il-10 and no or little IFN-γ. Anti-TCR antibody activation 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 Il-2 production. This could indicate either the ability of the hapten-immune $CD4^+$ T cells to produce Il-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 Il-2 and a Th2 population producing Il-4, Il-5, and Il-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 Il-4 and Il-10 to inhibit CHS responses have been well established (6, 7, 20–23). The direct role of Il-4 in regulating CHS has been further suggested in studies by Gautam and co-workers (20) in which anti–Il-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–Il-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 Il-4 (34–36), it is tempting to speculate that neutralization of Il-4 during sensitization may inhibit the development of the $CD4^+$ Th2 regulatory population during sensitization and subsequently result in increased CHS responses.

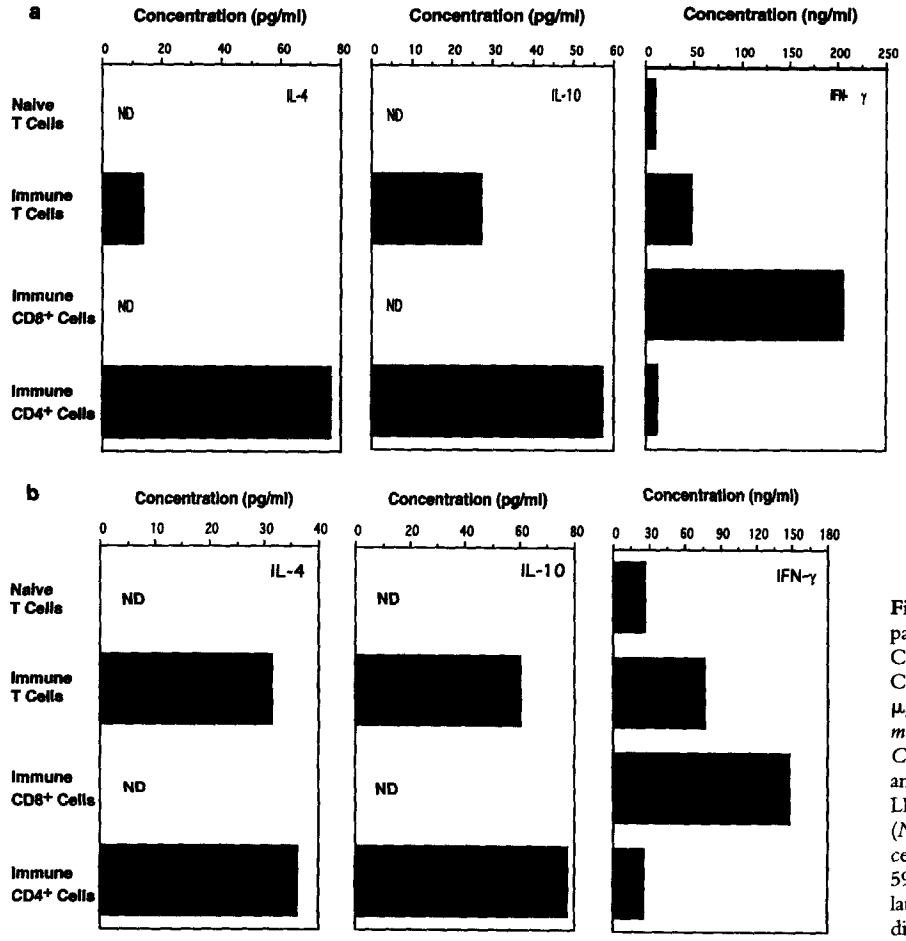

Figure 6. Hapten sensitization induces polarized patterns of cytokine production by $CD4^+$ and $CD8^+$ T cells in C57Bl/6 and B10.BR mice. C57Bl/6 (A) or B10.BR (B) mice were given 100 μg of rat IgG (*Immune T Cells*) or anti-CD4 (*Immune $CD8^+$ Cells*) or anti-CD8 (*Immune $CD4^+$ Cells*) mAb on three 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 2×10^5 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.

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 Il-10 were stimulated during culture of Ox-immune $CD4^+$ T cells on anti-TCR antibody, detectable Il-10 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,

41). Furthermore, results from D'Andrea et al. (42) have clearly shown the ability of Il-10 to inhibit mononuclear cell production of Il-12. Thus, the Il-10 produced by keratinocytes after hapten sensitization may render the LC incapable of producing Il-12 and unable to promote the induction of hapten-specific Th1 $CD4^+$ T cells. We have not addressed this issue directly but have recently tested the effect of exogenous Il-12 on the CHS response. The results have indicated that administration of Il-12 during hapten sensitization abrogates the induction of the Il-4–producing Th2 cells and instead induces an IFN-γ–producing (Th1) $CD4^+$ T cell population (DiIulio, N.A., H. Xu, and R.L. Fairchild, manuscript in preparation). This results in CHS responses with greater magnitude and prolonged kinetics than control responses, very similar to the response when the $CD4^+$ (Th2) regulatory cell population is absent. Although these observations are consistent with the hypothesis that LC do not produce Il-12 during the priming of the $CD4^+$ T cell population in CHS, the ability of LC to produce Il-12 after culture and/or stimulation in vitro or during other types of immune responses may differ from LC in CHS. Recently, Rook and coworkers (43) reported that stimulation of isolated LC with retinoic acid during overnight culture resulted in the production and secretion of the Il-12 heterodimer.

Although the segregated patterns of cytokine produced by hapten-primed $CD4^+$ and $CD8^+$ T cells in this study were virtually absolute, in many instances stimulation of CD8-deleted immune LNC resulted in the production of very small amounts of IFN-γ. At the present time it is not known if hapten-immune $CD4^+$ T cells are the source of this IFN-γ. The administration of anti-CD8 antibody, YTS 169, results in >96% deletion of $CD8^+$ T cells in our hands, and it is unlikely that the few remaining, if any, $CD8^+$ T cells make quantities of IFN-γ that are above the level of detection in the ELISA. It is possible that other cells, such as NK1.1 cells, produce IFN-γ after sensitization and may be the source of these small amounts of IFN-γ. A role for $NK1.1^+$ cells in producing cytokines such as IFN-γ and Il-4 to promote the generation of Th1 or Th2 $CD4^+$ T cell responses has been recently proposed and is under intensive study in several laboratories (44, 45).

Collectively, the results of this report have exposed the induction of two cellular components participating in the CHS response. The effector cells in the response are $CD8^+$ T (Tc1) cells producing IFN-γ, whereas Il-4/Il-10–producing $CD4^+$ T (Th2) cells function as negative regulators of the response. These results distinguish CHS from classical DTH responses, which are primarily, though not exclusively, mediated by IFN-γ–producing $CD4^+$ (Th1) T cells (10, 11). Studies in experimental murine cutaneous leishmaniasis have demonstrated the resistance of C57Bl/6 mice to infection through the ability to mount a Th1 $CD4^+$ T cell–mediated DTH response and clear the infection (29). Infection is lethal in BALB/c mice, which mount a Th2 $CD4^+$ response incapable of clearing the parasite load. In contrast to the disparate induction of Th1/Th2 responses to these parasite antigens, the induction of the effector $CD8^+$ and regulatory $CD4^+$ T cells in CHS does not appear to be dependent on the genetic background of the mouse strain under study. Hapten sensitization of C57Bl/6 and B10.D2 mice resulted in the generation of the Tc1 and Th2 populations of cytokine-producing cells. The induction of both of these cell populations during CHS results in an effector/regulatory balance with the Th2 $CD4^+$ T cells providing a protective role by dampening the response and preventing overt immunopathology during the CHS response. Disruption of the balance between the $CD4^+$ and $CD8^+$ T cells through either inhibition or deletion of the Th2 $CD4^+$ regulatory population would be predicted to result in extended immunopathology during the immune response to hapten-altered self-determinants. The delineation of the effector and regulatory mechanisms in CHS should provide the ability to skew the balance therapeutically in favor of the regulatory T cell population and control the magnitude and duration of the CHS response.

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