

CD8⁺ IL-17-Producing T Cells Are Important in Effector Functions for the Elicitation of Contact Hypersensitivity Responses¹

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Allergen-induced contact hypersensitivity (CHS) is a T cell-mediated delayed-type immune response which has been considered to be primarily mediated by CD8⁺ T cytotoxic type I (Tc1) cells. IFN- γ , the prototype Tc1 (Th1) cytokine, has been implicated as the primary inflammatory cytokine for CHS. In this study, we demonstrate that neutralization of IL-17 rather than IFN- γ suppresses the elicitation of CHS. The suppression does not result from inhibition of the proliferation of allergen-activated T cells. Allergen sensitization induces the development of distinct CD8⁺ T cell subpopulations that produce IFN- γ or IL-17. Although CD8⁺ IL-17-producing cells are stimulated by IL-23, they are inhibited by IL-12, a prototypical stimulator of IFN- γ -producing Tc1 cells. This indicates that CD8⁺ IL-17-producing cells are distinct from Tc1 cells and are important in effector functions at the elicitation of CHS. These studies provide insights into a novel mechanism for CHS. *The Journal of Immunology*, 2006, 177: 6852–6858.

Contact hypersensitivity (CHS)³ response is a T cell-mediated delayed-type hypersensitivity, which is induced by exposure to active allergens (haptens). For a long time, CHS, like protein Ag induced delayed hypersensitivity responses, has been considered to be mediated by Th1 T cytotoxic type I (Tc1) cells. In animal models, hapten-primed CD8⁺ T cells produce a large amount of IFN- γ , the prototype proinflammatory cytokine for Th1-type responses. CD4⁺ cells, in contrast, produce Th2 cytokines IL-4 and IL-10 (1). Correspondingly, CD8⁺ T cells are primary effector cells whereas CD4⁺ T cells play a minor role in CHS (1–3).

IL-17 is a group of inflammatory cytokines (4, 5). IL-17, also called IL-17A, is the prototype member of the family and can be produced by both CD4⁺ and CD8⁺ T cells (5). IL-17 is able to stimulate T cell proliferation and plays important roles in inflammatory diseases and autoimmune diseases (4–8). Strong evidence indicates that IFN- γ and IL-17 are produced by different subpopulations of CD4⁺ T cells, suggesting that CD4⁺ IL-17-producing cells (Th_{IL-17}) may be a specific subpopulation (7, 9, 10). Accumulating data have demonstrated that CD4⁺ Th_{IL-17} cells are primary effector cells in many autoimmune and allergic diseases that had been considered to be mediated by Th1 or Th2 cells (7, 8). Although most studies have been focused on characterization of

CD4⁺ Th_{IL-17} cells, less is known about the phenotype and function of CD8⁺ T cells that are able to produce IL-17.

The role of IL-17 in CHS has not yet been defined. However, clinical evidence indicates that some human T cell clones isolated from nickel allergic patients produce IL-17 (11). Nakae et al. (8) reported that CHS was reduced in IL-17 knockout mice compared with wild-type controls. Further analysis indicated that the activation of and IFN- γ production by CD4⁺ T cells were suppressed following hapten sensitization whereas CD8⁺ T cells were not affected. The authors concluded that IL-17 deficiency impaired the sensitization of hapten-specific CD4⁺ T cells. However, CD8⁺ T cells are primary effector cells and are able to elicit CHS independently of CD4⁺ T cells (1–3). It is unknown whether allergen sensitization induces the development of IL-17-producing T cells and whether IL-17 is an effector cytokine for the elicitation of CHS. Therefore, it remains to be determined whether the reduced CHS in the IL-17-deficient mice is due to lack of IL-17 production by CD8⁺ T cells even though they are normally activated.

The current report will specifically address the role of IL-17 in the elicitation of CHS and determine whether hapten sensitization induces the development of IL-17-producing T cells. Furthermore, subpopulations of hapten-specific CD4⁺ and CD8⁺ T cells that produce IL-17 and IFN- γ will be characterized. The outcome may unveil a new mechanism for the elicitation of CHS.

Materials and Methods

Animals and reagents

Female C57BL/6 mice (6–8 wk of age) were obtained from Charles River Laboratories. Rag-1^{−/−} mice were obtained from The Jackson Laboratory. All animal procedures were performed according to National Institutes of Health guidelines under protocols approved by the Institute Animal Care and Use Committee of the University of Alabama at Birmingham (Birmingham, AL).

Hybridoma lines GK1.5 (anti-CD4), Lyt-2 (anti-CD8), and XMGI.2 (anti-IFN- γ) were obtained from the American Type Culture Collection. Monoclonal rat anti-mouse IL-17 Ab (TC11–18H10) was purchased from Southern Biotechnology Associates. Abs used for flow cytometry, immunohistochemical staining, and ELISA were purchased from BD Pharmingen. Dinitrofluorobenzene (DNFB), dinitrobenzenesulfonic acid, sodium salt (DNBS), normal rat IgG, PMA, and ionomycin were obtained from Sigma-Aldrich. Recombinant mouse IL-17, IL-23, and IL-12 were obtained from R&D Systems.

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³ Abbreviations used in this paper: CHS, contact hypersensitivity; Tc1, T cytotoxic type I; DNFB, dinitrofluorobenzene; DNBS, dinitrobenzenesulfonic acid, sodium salt; DC, dendritic cell; BM-DC, bone marrow-derived DC.

Sensitization and elicitation of CHS

The induction and elicitation of CHS in mice were conducted as described (1). Briefly, mice were sensitized with DNFB on 2 consecutive days and challenged 5 days later. CHS was measured 24 h after challenge. To detect the role of IL-17 in the elicitation of CHS, mice were sensitized and treated twice i.p. with anti-IL-17 (200 μ g/mouse), anti-IFN- γ (400 μ g/mouse), or rat IgG (200 μ g/mouse) on days 4 and 5 after sensitization. The mice were challenged on day 5 and CHS was measured.

To detect whether neutralization of IL-17 induces prolonged effects on CHS, sensitized mice were treated with anti-IL-17, IFN- γ Abs, or rat IgG before challenge and CHS were measured as described above. These mice were rested for 3 wk and then rechallenged for measurement of CHS.

To examine transfer of CHS by primed T cells, primed CD8⁺ T cells from the draining lymph nodes of sensitized mice were purified and transferred i.v. into naive Rag-1-deficient mice (5 $\times$ 10⁶ cells/mouse). The recipient mice were treated twice with anti-IL-17 Ab or rat IgG 1 day before and on the day of cell transfer. The mice were challenged immediately following cell transfer and CHS was measured. As a control, Rag-1-deficient mice were sensitized and challenged. Naive controls were Rag-1-deficient mice that were neither transferred with primed T cells nor sensitized, but were challenged.

RT-PCR

The mRNA level was semiquantitatively measured by RT-PCR as described in previous studies (12). Briefly, total RNA was purified from mouse ear skin by using TRIzol according to manufacturer's instructions (Invitrogen Life Technologies). First-strand DNA was synthesized using an Omniscript Reverse Transcriptase kit (Qiagen) according to the instructions. PCR was conducted as routines. As controls, RNA samples without reverse transcription were subject to PCR to exclude DNA contamination. The following primers were applied (5'-3'): IL-1 α (289 bp), TCAGCAC CTTACACCTACC and GCAACTCCTTCAGCAACAC; IL-1 β (206 bp), ACAGCAGCACATCAACAAGAG and ATGGGAACGTCACAC ACCAG; IL-6 (202 bp), CCTCTCTGCAAGAGACTTCC and GCACAA CTCTTTTCTCATTTCC; KC (164 bp), GATTACCTCAAGAATCAG CAG and TGGGGACACCTTTTATGATC; IFN- γ (221 bp), ACAAT CAGGCCATCAGCAAC and TCAGCAGCGACTCCTTTTCC; IL-17 (300 bp), TCTCATCCAGCAAGAGATCC and GAATCTGCCTCTGAA TCCAC. PCR products were separated by agarose electrophoresis and results were recorded using a gel documentation system (Bio-Rad). The density of bands was measured using the software Quantity One (Bio-Rad). The relative expression level of each cytokine for each sample is calculated as density of cytokine/density of GAPDH.

Histology and immunohistochemical staining

To examine the histology of skin tissues, samples were collected 24 h after challenge and fixed in 10% formalin. Paraffin-embedded tissue sections were made and stained with H&E by a core facility at the university. Sections were evaluated by Dr. T. R. Schoeb (University of Alabama at Birmingham, Birmingham, AL).

To characterize leukocyte infiltration in skin, cryosections (5 μ M) were cut and fixed with cold acetone for 5 min. After rehydration in PBS, sections were incubated with an anti-CD16/CD32 Ab (2.4G/2) to block non-specific binding and then stained with PE-labeled CD3, FITC-labeled CD11b, and biotin labeled Gr-1 Abs (BD Pharmingen). The binding of biotin-labeled Gr-1 Ab was detected with streptavidin-Alexa 488 (BD Pharmingen). Sections were counterstained with fluorescence dye 4',6'-diamidino-2-phenylindole (DAPI). Pictures were taken microscopically with a $\times$ 10 objective. Positive cells were counted in 10 fields of each group (three mice). Average number of positive cells per field was calculated and the difference between groups was analyzed statistically.

T cell subpopulations

In vivo depletion of CD4⁺ or CD8⁺ T cells was performed by treatment of animals with specific Abs as described previously (1). Briefly, mice were treated by i.p. injection of 100 μ g of GK1.5, Lyt-2, rat IgG on 3 consecutive days. In vitro purification of hapten-primed CD4⁺ or CD8⁺ T cells was conducted using the MACS system according to the manufacturer's instruction (Miltenyi Biotec). The efficiency of depletion and purity of T cell subpopulations was determined by flow cytometry analysis using specific Abs to target cells.

In vitro measurement of cytokine production and T cell proliferation

Bone marrow-derived dendritic cells (BM-DC) were prepared and used for in vitro stimulation of primed T cells as described (1, 3, 13). DNFB-primed

T cells (2 $\times$ 10⁶/ml) were stimulated with DNBS-labeled BM-DC (2 $\times$ 10⁵/ml). Cytokine concentrations in culture supernatants were measured 48 h after cultures by cytokine-specific ELISA and T cell proliferation was determined 4 days after cultures by incorporation of [³H]thymidine.

Flow cytometry analysis

Intracellular staining of IFN- γ and IL-17 was conducted using the Cytofix/Cytoperm kit obtained from BD Pharmingen according to the manufacturer's instruction. Briefly, naive or DNFB-primed T cells were incubated with DNBS-labeled BM-DC for 48 h. The cells were then stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) overnight (10–12 h) in the presence of GolgiPlug. Our preliminary experiments showed that the overnight stimulation (4, 5, 6 h, and overnight) was necessary for detection of IFN- γ or IL-17-positive cells. The cells harvested after stimulation were stained with Alexa 488-labeled CD4⁺ or CD8⁺, PE-labeled anti-IL-17, and allophycocyanin-labeled IFN- γ Abs. To detect whether IL-17-producing T cells are $\alpha\beta$ T cells, cells were stained with Alexa 488-labeled CD4⁺ or CD8⁺, PE-labeled anti-IL-17, and allophycocyanin-labeled anti-TCR β -chain Ab (H57-597). The CD4⁺ or CD8⁺ T cells were gated and 10,000 events were collected in a flow cytometer (FACSCalibur; BD Biosciences).

Statistical analysis

The differences between experimental groups were analyzed using the Student *t* test with *p* < 0.05 being considered statistically significant.

Results

Neutralization of IL-17 suppresses the elicitation of CHS responses

A defect in the activation of hapten-primed CD4⁺ T cells has been considered as a mechanism for reduced CHS in IL-17 knockout mice (8). It is, therefore, unknown whether IL-17 has effector functions during the elicitation of CHS. To examine it, sensitized mice were treated with anti-IL-17 Ab before challenge and the effect on CHS was measured. The result indicated that treatment with

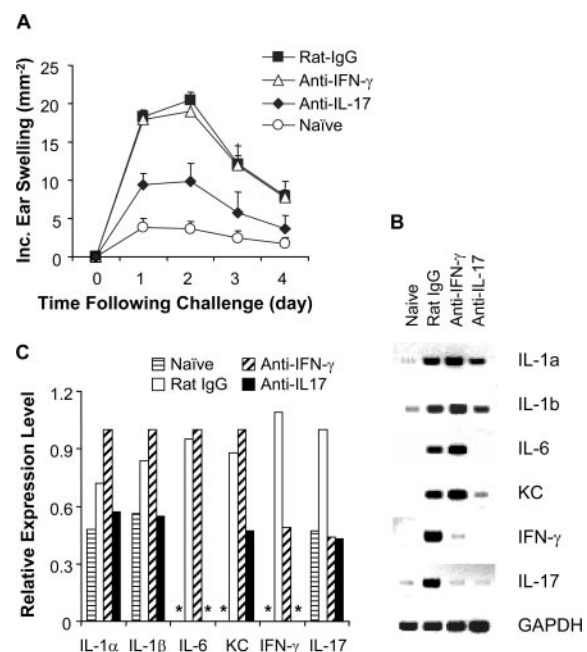


FIGURE 1. Neutralization of IL-17 suppresses the elicitation of CHS. **A**, Sensitized mice were treated with anti-IL-17, anti-IFN- γ Ab, or normal rat IgG before challenge. Naive mice that were challenged served as negative controls. The difference between anti-IL-17 with anti-IFN- γ or rat IgG-treated mice is significant at all time points (*p* < 0.05). **B**, The expression of cytokines and chemokines in ear tissues was determined by RT-PCR. GAPDH served as a housekeeping control. **C**, Relative expression level of each cytokine for each sample is calculated as density of cytokine/density of GAPDH. *, Undetectable.

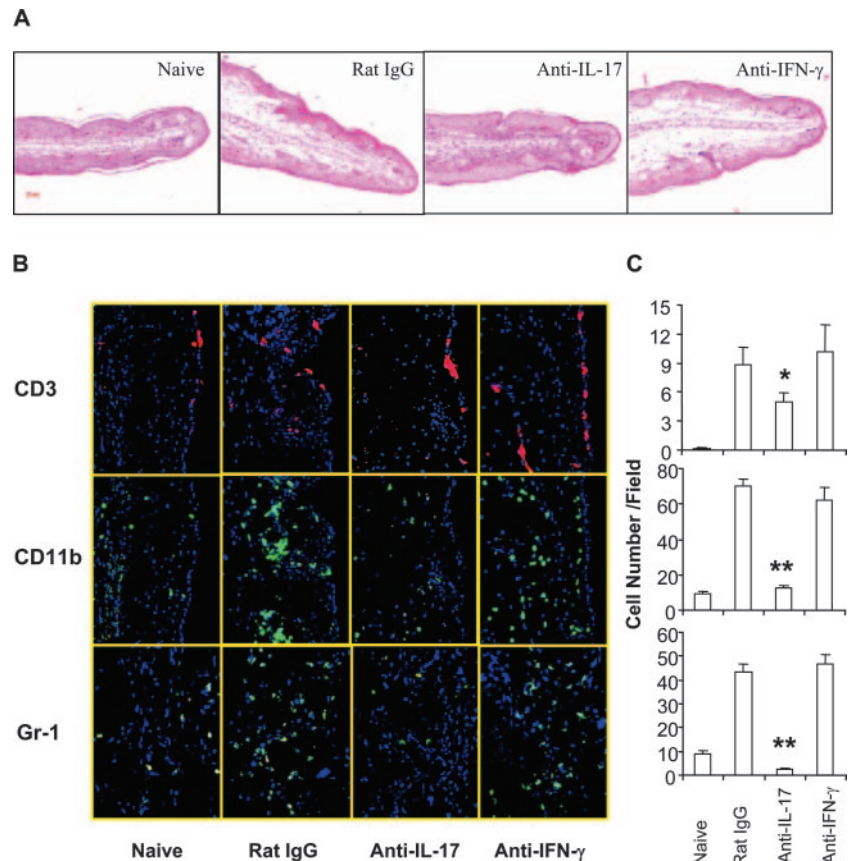


FIGURE 2. Neutralization of IL-17 reduces edema and leukocyte infiltration in the skin. Ear skin samples were collected 24 h after challenge and were subject to histology analysis and immunohistochemical staining. **A**, Histology of ear skin. Paraffin-embedded sections were stained with H&E and pictures were taken microscopically with a $\times 10$ objective. The bar represents 50 μ M. **B**, Immunohistochemical staining shows CD3⁺ T cells (red), CD11c⁺ monocytes/macrophages (green), and Gr-1⁺ granulocytes (green) in skin samples. The sections were counterstained with fluorescence dye DAPI (blue). Pictures were taken microscopically with a $\times 20$ objective. **C**, Positive cells were counted in 10 fields of each group. The values are shown as mean $\pm$ SEM of three mice per group (*, $p < 0.05$; **, $p < 0.01$). The data are representative of two to three independent experiments.

anti-IL-17 (200 μ g/mouse) inhibited CHS compared with controls treated with normal rat IgG at the same dose (Fig. 1A, $p < 0.05$). In contrast, treatment of mice with a doubled amount (400 μ g/mouse) of a neutralizing anti-IFN- γ Ab (XMG1.2) did not show any effect.

To examine mechanisms for the suppression of CHS in anti-IL-17-treated mice, a panel of inflammatory cytokines which are known to regulate CHS was examined. Results showed that the expression of IL-1 α and β , IL-6, IFN- γ , IL-17, and KC (a chemokine for neutrophils) was low to undetectable in naive mice that were challenged but not sensitized (Fig. 1B). The level of the cytokines in anti-rat IgG-treated samples was increased compared with naive controls. The level of the cytokines in the anti-IL-17-treated sample was lower than that in rat IgG-treated samples (Fig. 1, B and C). The treatment with anti-IFN- γ Ab did not have an effect on IL-1, IL-6, and KC expression but reduced the level of IFN- γ and IL-17 compared with rat IgG-treated samples. These experiments were repeated three times and similar results were observed.

Neutralization of IL-17 reduces edema and leukocyte infiltration in the hapten-challenged skin tissue

The histology analysis indicated that a low level of edema was observed in the skin of naive control mice that were not sensitized but challenged (Fig. 2A). The edema and ear thickness were increased in the skin of rat IgG-treated mice compared with naive controls. The treatment with anti-IL-17 Ab reduced edema and thickness compared with rat IgG treatment whereas the anti-IFN- γ -treated sample was similar to rat IgG control.

To examine whether anti-IL-17 treatment inhibited the infiltration of T cells, monocytes/macrophages and granulocytes, hapten-challenged ear tissues were stained with anti-CD3, CD11b, and

Gr-1 Abs. Results indicated that a few CD3⁺, CD11b⁺, and Gr-1⁺ cells could be detected in naive controls that were challenged but not sensitized. In the mice treated with rat IgG, CD3⁺, CD11b⁺, and Gr-1⁺ cells were increased (Fig. 2B). The treatment with anti-IL-17 Ab significantly decreased the number of CD3, CD11b, and Gr-1-positive cells whereas the treatment with anti-IFN- γ Ab showed little effect compared with rat IgG control (Fig. 2, B and C, $p < 0.05$).

Hapten-primed T cells produce IL-17

It has not yet been documented whether hapten sensitization induces the development of T cells that are able to produce IL-17 although some T cell clones isolated from allergic patients have been shown to produce the cytokine (11). In our initial experiments, primed T cells were isolated from the draining lymph nodes of DNFB-sensitized mice and placed in cultures with DNBS-labeled BM-DC. Hapten-labeled BM-DC stimulated the production of IL-17 and IFN- γ by primed, but not naive, T cells (Fig. 3A). Treatment of BM-DC with LPS, which induced activation and maturation of DC, significantly enhanced the production of IL-17 and IFN- γ compared with untreated BM-DC (Fig. 3A, $p < 0.05$). Additional experiments showed that both primed CD4⁺ and CD8⁺ T cells could produce IL-17. However, CD8⁺ T cells produced a higher level (~ 2 times) than CD4⁺ T cells (Fig. 3B, $p < 0.05$). Additionally, CD8⁺ T cells produced ~ 10 times more IFN- γ than CD4⁺ T cells, a phenomenon observed in previous studies (1).

Treatment of BM-DC increased the production of IL-12 and IL-23 (data not shown). IL-12 and IL-23 have been reported to regulate Th1 (Tc1) and Th_{IL-17} cells, respectively (7, 14, 15). In our experiments, addition of exogenous IL-23 (5 ng/ml) significantly increased the production of IL-17 by primed CD4⁺ and CD8⁺ T cells while it had little effect on IFN- γ production. In

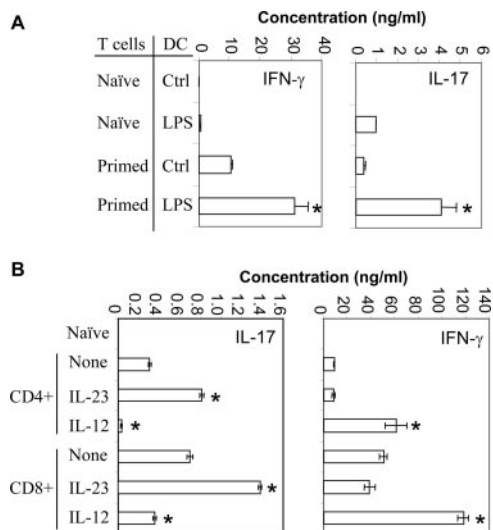


FIGURE 3. Production of IL-17 by hapten-primed T cells. Primed T cells were placed in cultures with hapten-labeled BM-DC for 48 h and cytokines in supernatants were measured by ELISA. *A*, Hapten-primed T cells produce IL-17 and IFN- γ . Treatment with LPS increased the ability of BM-DC (LPS) to stimulate IL-17 and IFN- γ production compared with untreated controls (Ctrl) (*, $p < 0.05$). Naïve T cells served as controls. The data (mean $\pm$ SD) are representative of three independent experiments. *B*, Effects of IL-12 and IL-23 on IL-17 production. CD4⁺ and CD8⁺ primed T cells were purified and cultured with hapten-labeled BM-DC in the absence or presence of IL-12 or IL-23. The data (mean $\pm$ SD) are representative of three independent experiments. *, $p < 0.05$, compared with controls (None).

contrast, exogenous IL-12 (5 ng/ml) significantly inhibited IL-17 production whereas increased IFN- γ production by both T cell subpopulations (Fig. 3*B*, $p < 0.05$). The result suggests that IL-17 may not be produced by classical Th1 or Tc1 cells.

Distinct subpopulations of hapten-primed T cells produce IL-17 or IFN- γ

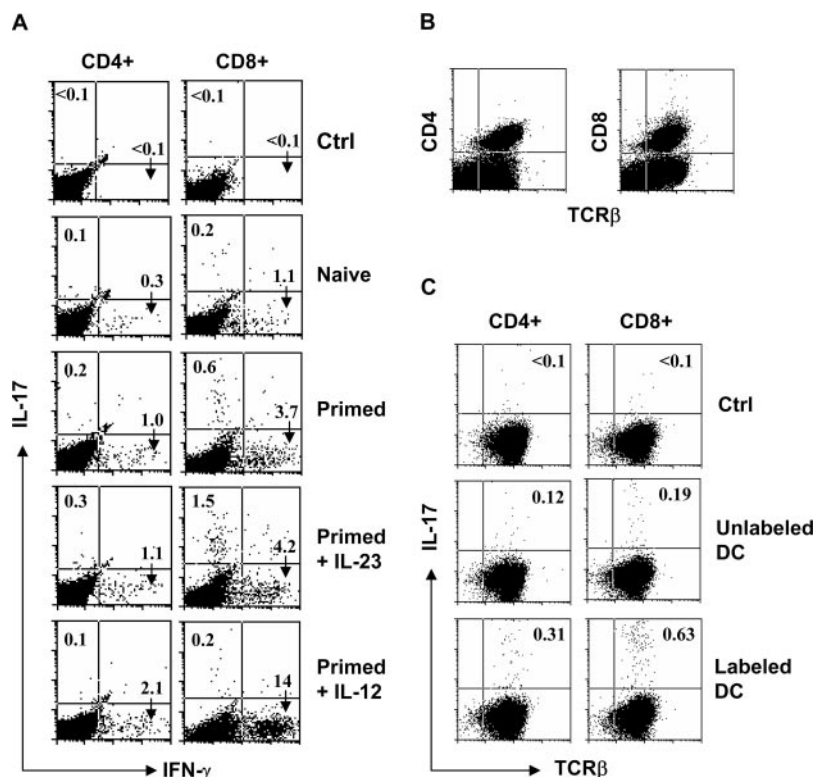
To further characterize T cell subsets that produce IFN- γ and IL-17, hapten-primed T cells were double stained with anti-IL-17 and IFN- γ Abs and cytokine profiles of CD4⁺ and CD8⁺ T cells were analyzed. Results showed that different subsets of hapten-primed CD4⁺ and CD8⁺ T cells produced IL-17 or IFN- γ (Fig. 4*A*). Addition of exogenous IL-23 in cultures increased IL-17-producing cells compared with the controls while it had a minimal effect on IFN- γ -producing cells. In contrast, addition of IL-12 in cultures reduced the percentage of IL-17-producing T cells but greatly increased IFN- γ -positive cells.

There were reports that other cells apart from T cells could produce IL-17 as well (5, 16). To determine whether IL-17-producing CD4⁺ and CD8⁺ T cells from hapten-sensitized mice were $\alpha\beta$ T cells, an anti-TCR β -chain Ab was used. Results showed that most of CD4⁺ and CD8⁺ T cells from draining lymph nodes were positive for TCR β -chain (Fig. 4*B*). CD4⁺ or CD8⁺ T cells that were positive for IL-17 were stained by anti-TCR β -chain Ab (Fig. 4*C*). This implicates that in CHS, IL-17-producing CD4⁺ or CD8⁺ cells are $\alpha\beta$ T cells. Stimulation of primed T cells with DC that were not labeled with hapten gave rise to a background level of IL-17-positive cells as seen in the controls with naïve T cells (<0.2%, Fig. 4, *A* and *C*).

CD8⁺ IL-17-producing T cells are important for the elicitation of CHS responses

IL-17 deficiency impairs the activation of hapten-specific CD4⁺ but not CD8⁺ T cells (8). To determine the role of CD4⁺ and CD8⁺ T cells in CHS induced by DNFB, mice were depleted of CD4⁺ or CD8⁺ T cells before hapten sensitization and the effect on CHS was measured following challenge. Flow cytometry analysis of draining lymph node cells showed that the Ab treatment resulted in almost complete depletion of the target T cell subset (Fig. 5*A*). Depletion of CD4⁺ T cells significantly increased

FIGURE 4. Characterization of hapten-primed CD4⁺ and CD8⁺ T cells that produce IL-17 and IFN- γ . *A*, Cells were stained with anti-CD4 or CD8 (Alexa 488), IL-17 (PE), and IFN- γ (allophycocyanin) Abs. Control samples (Ctrl) were stained with PE and allophycocyanin-labeled isotype controls. The CD4⁺ or CD8⁺ T cells were gated and IL-17 or IFN- γ -positive cells were analyzed. *B*, Cells were stained with anti-CD4 or CD8 (Alexa 488) and TCR β -chain Ab (allophycocyanin). *C*, Cells were stained with anti-CD4 or CD8 (Alexa 488), IL-17 (PE), and TCR β -chain (allophycocyanin) Abs. Primed T cells that were stimulated with BM-DC without hapten labeling (Unlabeled DC) served as controls for those stimulated with hapten-labeled DC (Labeled DC). CD4⁺ or CD8⁺ T cells were gated for analysis. The numbers indicate the percentage of IFN- γ or IL-17-positive cells in the gated CD4⁺ or CD8⁺ cell population. The data are representative of three to four independent experiments.



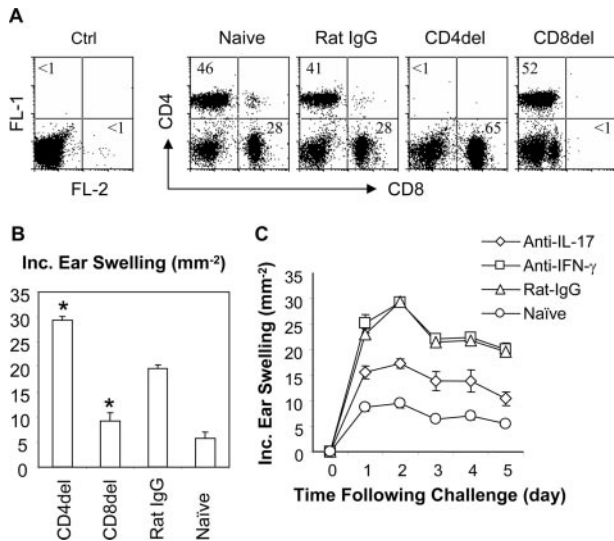


FIGURE 5. The role of IL-17 in CD8⁺ T cell-mediated CHS. *A*, Mice were depleted of CD4⁺ (CD4del) or CD8⁺ (CD8del) T cells by *in vivo* injection of specific Abs. The efficiency of depletion was determined by flow cytometry analysis of the draining lymph node cells. Numbers indicate percentages of positive cells. *B*, Mice were depleted of CD4⁺ (CD4del) or CD8⁺ (CD8del) T cells and then sensitized. CHS was measured following challenge. *, $p < 0.05$. *C*, Mice were depleted of CD4⁺ T cells and sensitized. The mice were then treated before challenge with anti-IL-17, IFN- γ Ab, or control rat IgG. The treatment with anti-IL-17 Ab significantly reduces CHS at all time points ($p < 0.05$). The data are shown as mean $\pm$ SD of three to four mice per group and are representative of two to three independent experiments.

whereas depletion of CD8⁺ T cells significantly reduced CHS (Fig. 5*B*, $p < 0.05$).

To examine whether neutralization of IL-17 inhibited CD8⁺ T cell-mediated CHS, mice were depleted of CD4⁺ T cells before sensitization and then treated with anti-IL-17, anti-IFN- γ , or rat IgG before challenge as described above. The treatment with anti-IL-17 Ab significantly inhibited the response compared with controls in a similar manner observed in normal mice (Fig. 5*C*, $p < 0.05$). Again, administration of anti-IFN- γ Ab did not have significant effects on the response.

To further confirm the role of IL-17-producing CD8⁺ T cells in CHS, primed CD8⁺ T cells were purified and transferred into Rag-1-deficient mice that were treated with anti-IL-17 Ab or rat IgG. Transfer of primed CD8⁺ T cells elicited CHS following challenge of mice that were treated with rat IgG. In contrast, the treatment with anti-IL-17 Ab significantly inhibited CHS (Fig. 6, $p < 0.05$). Sensitization of Rag-1-deficient mice did not induce significant levels of CHS compared with control naive mice that were not sensitized but challenged. This indicates that the CHS was induced by transferred T cells in Rag-1-deficient recipient mice.

Neutralization of IL-17 does not affect primed T cells

IL-17 has been reported to regulate the proliferation of T cells and maturation of DC (6, 17). To examine whether the reduced CHS in mice treated with anti-IL-17 Ab might result from suppression of primed T cells, primed CD4⁺ and CD8⁺ T cells were purified and cultured with hapten-labeled BM-DC in the presence of anti-IL-17 Ab. Preliminary experiments showed that IL-17 was completely neutralized (undetectable) in cultures with 5 μ g/ml of the Ab as assessed by ELISA (data not shown). Neutralization of IL-17 did not have any significant effect on the proliferation of primed CD4⁺ and CD8⁺ T cells compared with controls (Fig. 7*A*).

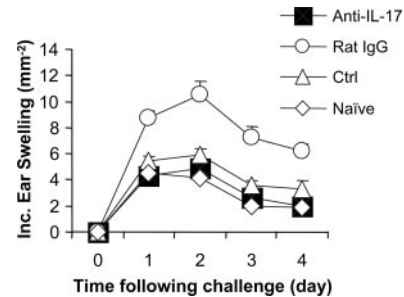


FIGURE 6. The role of IL-17-producing CD8⁺ T cells in transfer of CHS. Primed CD8⁺ T cells were purified by using MACS system and transferred *i.v.* into naive Rag-1-deficient mice. The mice were treated with anti-IL-17 or rat IgG before challenge and CHS was measured. As a control (Ctrl), Rag-1-deficient mice were sensitized and challenged. Mice that were neither transferred with cells nor sensitized were challenged and served as naive control (Naive). The difference between rat IgG and anti-IL-17 Ab-treated group is significant at all indicated time points ($p < 0.05$). The data are shown as mean $\pm$ SD of three mice per group and are representative of two independent experiments.

To further determine whether the anti-IL-17 Ab treatment directly inhibited hapten-primed T cells, sensitized mice were treated with anti-IL-17 Ab before hapten challenge and CHS were measured as described. The mice were then rested for 3 wk, when the ear thickness returned to background levels. These mice were then rechallenged and CHS was measured. In contrast to the reduced CHS following the first challenge (Fig. 1*A*), the CHS following rechallenge was identical in mice treated with anti-IL-17, anti-IFN- γ Abs or rat IgG (Fig. 7*B*). Similar results were observed in Rag-1-deficient mice following rechallenge, which were transferred with primed CD8⁺ T cells and treated with rat IgG or anti-IL-17 Ab (data not shown). This indicates that primed T cells are not affected by neutralization of IL-17 and are able to elicit CHS following rechallenge.

Discussion

Recent studies have demonstrated that CD4⁺ IL-17-producing T cells play important roles in inflammatory and autoimmune diseases. However, less is known about the development of CD8⁺ IL-17-producing T cells and their roles in immune responses. In this study, we demonstrate a new mechanism for CHS, which is mediated by CD8⁺ IL-17-producing T cells. This CD8⁺ IL-17-producing T cell subpopulation is distinct from CD8⁺ IFN- γ -producing Tc1 cells and is important in effector functions during the elicitation of CHS.

Nakae et al. (8) reported that CHS was reduced in IL-17-deficient mice. Further analysis indicated that IL-17 deficiency impaired the activation of CD4⁺ but did not affect the function of CD8⁺ T cells and DC. Transfer of primed wild-type T cells elicited similar levels of CHS in IL-17-deficient and wild-type recipient animals, suggesting that IL-17 deficiency might not affect the elicitation of CHS. However, our results demonstrate that IL-17 is an important inflammatory cytokine for the elicitation of CHS. One explanation for the discrepancy is that in the reported studies, wild-type T cells that were transferred into IL-17-deficient mice might produce IL-17 and elicit CHS in the IL-17-deficient recipient mice. It is supported by our results indicating that IL-17 is produced by hapten-primed T cells and that neutralization of IL-17 inhibits the transfer of CHS by hapten-primed T cells in Rag-1-deficient recipient mice.

The suppression of CHS in mice treated with anti-IL-17 Ab results from decrease of inflammatory cytokines and reduction of

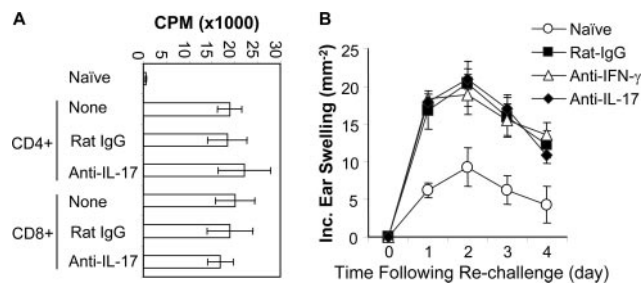


FIGURE 7. Neutralization of IL-17 does not affect primed T cells. *A*, Neutralization of IL-17 has no effect on the proliferation of primed T cells. Primed CD4⁺ or CD8⁺ T cells were purified and cultured with hapten-labeled BM-DC in the presence of anti-IL-17 Ab or rat IgG. The data (mean $\pm$ SD) are representative of three independent experiments. *B*, Neutralization of IL-17 does not affect CHS following rechallenge (details in *Materials and Methods*). There is no significant difference between all treated groups of mice following rechallenge. The data (mean $\pm$ SD) are representative of two independent experiments.

leukocyte infiltration in the hapten-challenged skin tissues. IL-1, IL-6, and KC have been reported to be important inflammatory cytokines and chemokines for the elicitation of CHS (18–21). In anti-IL-17-treated mice, the expression of IL-1 α , IL-1 β , IL-6, and KC was down-regulated compared with rat IgG-treated controls. Although this may be a part of mechanisms for the suppression of CHS in anti-IL-17-treated mice, it has to be noted that IL-17 is able to regulate the production of many cytokines and chemokines and the expression of adhesion molecules (5, 20, 22–26). Therefore, it is quite possible that in addition to the ones tested in the current studies, other cytokines may be regulated by neutralization of IL-17. The treatment with anti-IFN- γ Ab did not affect the expression of IL-1, IL-6, and KC whereas it reduced the expression of IFN- γ and IL-17 mRNA in the challenged skin. It suggests that anti-IFN- γ treatment might have some effects even though it did not affect leukocyte infiltration and CHS. Although literature indicate that neutralization of IFN- γ enhances IL-17 production by CD4⁺ T cells (9, 10), effects of IFN- γ on other cells that are able to produce IL-17 remain unknown. Previous studies reported controversial results about the effect of IFN- γ on CHS in IFN- γ or IFN- γ receptor-deficient mice (27–30). Given important roles of IFN- γ in many inflammatory diseases, we are investigating this issue in our ongoing studies.

The role of IL-17 in the regulation of neutrophil infiltration in inflammatory reactions has been reported (24, 25). In CHS, neutralization of IL-17 significantly inhibits the infiltration of Gr-1⁺ granulocytes in the hapten-challenged skin. Additionally, the infiltration of CD3⁺ T cells and CD11b⁺ monocytes/macrophages is inhibited as well. It is unknown whether IL-17 has direct chemoattractive effects on these leukocytes. Likely, the down-regulation of cytokines and chemokines might be attributed to the decrease of leukocyte infiltration in anti-IL-17-treated mice. Certainly, further studies are required to determine mechanisms for IL-17-mediated regulation of leukocyte migration and to validate the role of the cytokines in IL-17-mediated inflammatory reactions in CHS.

Although both hapten-primed CD4⁺ and CD8⁺ T cells are able to produce IL-17, CD8⁺ T cells produce a higher level of IL-17 than CD4⁺ T cells. This supports the major role of CD8⁺ T cells in the elicitation of CHS (1–3). Similar to other models (7, 9, 10), hapten-primed CD4⁺ Th_{IL-17} are distinct from Th1 cells. Moreover, primed CD8⁺ IL-17-producing cells are distinct from IFN- γ -producing cells (Tc1) as well. This CD8⁺ subset has not been previously reported and will be designated as CD8⁺ T_{IL-17}. It is to note that in CHS model, CD8⁺ T cells are major IFN- γ producers.

A small portion of CD4⁺ T cells can produce IFN- γ as evidenced by ELISA and intracellular cytokine staining (Figs. 3 and 4). It is in accordance with our previous studies indicating that ~0.3% of primed CD8⁺ T cell-depleted lymph node cells were positive for IFN- γ as assessed by ELISPOT (31). This is a unique feature of CHS compared with many other inflammatory disease models that are primarily mediated by CD4⁺ T cells.

Effects of IL-23 on IL-17 production by CD4⁺ T cells have been reported (7, 14). Our results indicate that IL-23 stimulates whereas IL-12 inhibits CD8⁺ T_{IL-17} cells in CHS. This implicates that different mechanisms modulate CD8⁺ Tc1 and T_{IL-17} cells. It is somewhat surprising that IL-12 inhibits CD8⁺ T_{IL-17} cells because IL-12 has been reported to enhance CHS (32). However, another study demonstrated that CHS was not compromised in IL-12 knockout mice (15). Further studies are required to define the role of IL-12 and IL-23 in the regulation of hapten-primed T cells and CHS. The mechanism for IL-12-mediated inhibition of IL-17-producing cells has yet to be defined. Two recent reports indicate that IFN- γ inhibits the development of CD4⁺ IL-17-producing T cells (9, 10). It is possible that the high level of IFN- γ in the cultures with exogenous IL-12 inhibits IL-17 production by hapten-activated T cells.

A line of studies including ourselves have demonstrated that CD8⁺ T cells are primary effector cells whereas CD4⁺ T cells are less effective in CHS (1–3, 33, 34). It is confirmed in the current studies that CHS is reduced in CD8⁺-depleted, whereas increased in CD4⁺ T cell-depleted, mice compared with untreated controls (Fig. 5B). However, a report showed that CHS was reduced in CD4⁺ T cell knockout mice compared with wild-type controls, suggesting that both CD4⁺ and CD8⁺ T cells might be required for optimal responses (35). Interestingly, a recent study has found that in CD4⁺ T cell knockout mice, a CD4-negative regulatory cell population develops, which suppresses the function of CD8⁺ T cells in CHS. The development of the CD4⁺ regulatory cells is restricted by MHC class II molecules, and CHS responses in MHC class II-deficient mice, which are also deficient in CD4⁺ T cells, are increased compared with wild-type controls (36).

Hapten-primed CD8⁺ IL-17-producing T cells are primary effector cells for CHS although it is not excluded that IL-17 derived from other cell types may play a role. This is supported by facts indicating that 1) neutralization of IL-17 suppresses CHS in mice depleted of CD4⁺ T cells; 2) transfer of primed CD8⁺ T cells into Rag-1^{-/-} mice elicits CHS which is inhibited by neutralization of IL-17; 3) hapten-primed IL-17-producing CD8⁺ cells are $\alpha\beta$ T cells.

The suppression of CHS by neutralization of IL-17 might not result from inhibition of T cell proliferation because blockade of IL-17 did not have significant effects on the growth of hapten-primed T cells stimulated by hapten-labeled DC. Moreover, anti-IL-17-treated mice could develop an identical level of CHS as control mice when they were rechallenged after 3 wk rest. It implicates that functions of primed T cells in anti-IL-17-treated mice are not affected by the neutralization of IL-17 and that the reduced CHS results from blockade of IL-17-mediated effects on inflammatory reactions. Yao et al. (6) reported that IL-17 enhanced T cell proliferation induced by Con A, PHA, or anti-TCR Abs. These reagents are polyclonal stimuli for T cells and mediate stronger signals than hapten-specific stimulation mediated by DC in our experiments. Nakae et al. (8) showed that in IL-17 knockout mice, the activation of hapten-primed CD8⁺ T cells was not affected although primed CD4⁺ T cells were inhibited. Moreover, our experiments examined the effect of IL-17 on hapten-primed T cells. This is different from the reported studies, which examined effects of IL-17 on the activation of resting T cells. In fact, Nakae et al.

(8) concluded in their studies that IL-17 affected only the sensitization of CD4⁺ T cells in CHS. It is to note that the level of CHS following the rechallenge is similar to that after the first challenge. An explanation is that effector cells go back to the memory status after recession of CHS following the first challenge. The memory cell level at 3 wk after the first challenge may be similar to that after the sensitization. Antonysamy et al. (17) reported that IL-17 induced maturation of DC and increased the ability of DC to stimulate T cells. However, it had little effect on mature DC. Our experiments used LPS-induced mature DC which might not be regulated by IL-17. Moreover, the migration and maturation of DC have been found to be normal in IL-17-deficient mice (8).

In summary, IL-17 is an important inflammatory cytokine for the elicitation of CHS. Hapten-primed CD8⁺ T_{IL-17} cells are distinct from IFN- γ -producing cells (Tc1) and are primary effector cells for the response. CD8⁺ T_{IL-17} cells are stimulated by IL-23 but inhibited by IL-12, implicating that different mechanisms regulate the development and function of CD8⁺ Tc1 and T_{IL-17} cells. These data provide insights into a novel mechanism for the elicitation of CHS and may lead to new strategies for the treatment of allergen-induced contact dermatitis.

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Disclosures

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