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Antigen-Specific T Cell Sensitization Is Impaired in IL-17-Deficient Mice, Causing Suppression of Allergic Cellular and Humoral Responses

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Summary

Interleukin-17 (IL-17) is a proinflammatory cytokine produced by T cells. The involvement of IL-17 in human diseases has been suspected because of its detection in sera from asthmatic patients and synovial fluids from arthritic patients. In this study, we generated IL-17-deficient mice and investigated the role of IL-17 in various disease models. We found that contact, delayed-type, and airway hypersensitivity responses, as well as T-dependent antibody production, were significantly reduced in the mutant mice, while IL-17 deficiency of donor T cells did not affect acute graft-versus-host reaction. The results suggest that impaired responses were caused by the defects of allergen-specific T cell activation. Our findings indicate that IL-17 plays an important role in activating T cells in allergen-specific T cell-mediated immune responses.

Introduction

IL-17 is a T cell-derived proinflammatory cytokine originally named cytotoxic T lymphocyte-associated serine esterase-8 (CTLA-8) (Rouvier et al., 1993). Murine IL-17 is a 21 kDa glycoprotein, consisting of 147 amino acids, which has a 63% amino acid homology with human IL-17 (155 amino acids) (Yao et al., 1995b). It has no obvious homology with other cytokines but has a 57% homology with the predicted amino acid sequence of the open reading frame 13 (ORF13) of *Herpesvirus saimiri* (HVS) (also called vIL-17) (Rouvier et al., 1993).

Recently, human IL-17 has been found to be included in a novel cytokine family consisting of IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F in addition to IL-17 and vIL-17; their identity with IL-17 is 16%–50% (Aggarwal and Gurney, 2002). A receptor for IL-17 (IL-17R) has been identified and found to share no homology with any other cytokine receptor family. Its mRNA expression shows a ubiquitous tissue distribution (Yao et al., 1995a). Although IL-17R has no obvious motifs in the intracellular domain, its signal transduction is shown to be mediated by tumor necrosis factor-associated factor 6 (TRAF6) but not by TRAF2 (Schwandner et al., 2000).

IL-17 is produced by TCRα/β^{+}CD4^{-}CD8^{-} thymocytes, as well as activated CD4^{+} and CD4^{+}CD45RO^{+} memory T cells (Yao et al., 1995b). Although activated CD8^{+} and CD8^{+}CD45RO^{+} memory T cells are also known to produce IL-17 in humans (Shin et al., 1999), its expression is restricted to CD4^{+} T cells in mice (Infante-Duarte et al., 2000). The expression in Th1 and Th2 cells seems to be different depending upon the conditions. While Aarvak et al. reported that IL-17 is produced by Th1/Th0 cells but not by Th2 cells, Albanesi et al. reported that both Th1 and Th2 cell clones from human skin-derived nickel-specific T cells produced IL-17 (Aarvak et al., 1999; Albanesi et al., 2000). On the other hand, IL-17 is produced by T cells expressing TNF-α but not by Th1 or Th2 cells in mice (Infante-Duarte et al., 2000).

IL-17 has pleiotropic activities including induction of TNF-α, IL-1β, IL-6, IL-8, G-CSF, and MCP-1 on various types of cells (Fossiez et al., 1998; Jovanovic et al., 1998), upregulation of ICAM-1 and HLA-DR expression on kerationocytes (Albanesi et al., 1999), induction of *iNOS* and cyclooxygenase-2 (*cox-2*) on chondrocytes (Shalom-Barak et al., 1998), induction of cox-2-dependent PGE2-mediated osteoclast differentiation factor (ODF) expression on osteoblasts (Kotake et al., 1999), and promotion of SCF- and G-CSF-mediated granulopoiesis (Schwarzenberger et al., 2000). IL-17 acts on T cells as a costimulatory factor (Yao et al., 1995a), enhances allorejection via promotion of dendritic cell (DC) maturation (Antonysamy et al., 1999), and promotes tumor rejection by activation of NK cells (Hirahara et al., 2000). IL-17 has also been detected in the sera as well as the diseased organs and tissues of various patients, suggesting its involvement in the development of various human diseases such as rheumatoid and Lyme arthritis and osteoarthritis (Attur et al., 1997; Aarvak et al., 1999; Infante-Duarte et al., 2000), multiple sclerosis (Matusevicius et al., 1999), systemic lupus erythematosus (Wong et al., 2000), allograft rejection (Antonysamy et al., 1999), and asthma (Wong et al., 2001). Furthermore, IL-17 has been shown to be involved in the host defense mechanism against *Klebsiella pneumoniae* infection by using IL-17R$^{-/-}$ mice (Ye et al., 2001). Neither the exact pathophysiologic role of IL-17 nor its mechanisms of action in the immune system, however, have been completely elucidated.

In this study, we generated IL-17-deficient (IL-17$^{-/-}$) mice in order to elucidate the roles of IL-17 in various inflammatory diseases and immune responses. Using this model, we have shown that IL-17 is involved in diseases such as contact hypersensitivity (CHS), delayed-type hypersensitivity (DTH), and airway hypersen-

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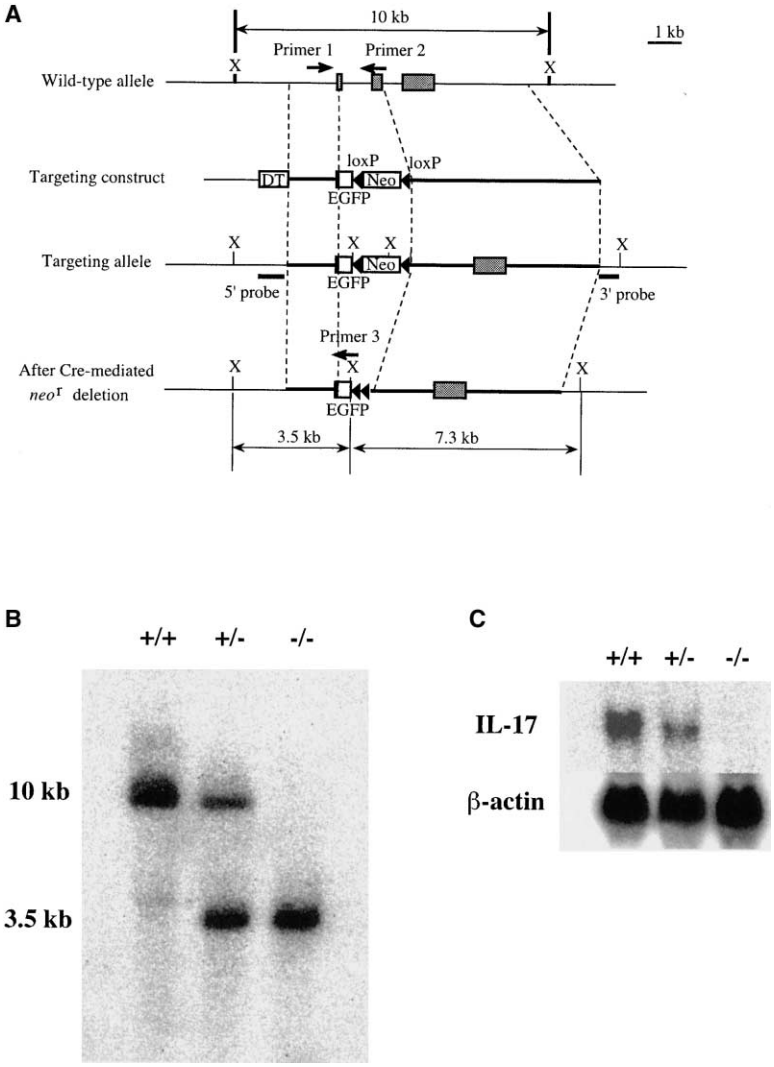


Figure 1. Generation of IL-17$^{-/-}$ Mice

(A) Structure of the mouse *il-17* locus (Wild-type allele), the IL-17 targeting construct (Targeting construct), and the predicted mutated *il-17* gene before (Targeting allele) and after neomycin resistance gene (*neo*^{r}) deletion (After Cre-mediated *neo*^{r} deletion). Exons are represented by boxes. Exon 1 and 2 of the *il-17* gene were replaced with the *EGFP* gene and the *neo*^{r} gene flanked by lox P sequences. A *DT* gene was attached to the 5′ end of the genomic fragment for negative selection. The external homologous regions shown in the targeting allele were used as the genomic probe for Southern blot analysis. Southern blot analysis for targeted clone screening was carried out using XbaI (X). Primers 1, 2, and 3 were used for genotyping of mice.

(B) Southern blot analysis of the thymus DNA from IL-17 wild-type (+/+), heterozygous (+/−), and mutant (−/−) littermates. The endogenous (10 kb) and targeted (3.5 kb) bands were shown using the 5′ probe.

(C) Northern blot analysis of the IL-17 mRNA from splenic T cells stimulated with plate-coated anti-CD3 plus anti-CD28 mAb for 48 hr.

sitivity response (AHR), but not in acute graft-versus-host reaction (GVHR), through activation of T cell priming.

Results

Generation of IL-17-Deficient Mice

We generated IL-17$^{-/-}$ mice by replacing the exon 1 and 2 of the *il-17* gene with a neomycin resistance gene (Figure 1A). Correct targeting of the IL-17 locus was confirmed by genomic Southern blot analysis (Figure 1B). The expression of IL-17 mRNA was not detected by Northern blot analysis in splenic T cells from IL-17$^{-/-}$ mice stimulated with plate-coated anti-CD3 plus anti-CD28 mAb (Figure 1C). The levels of IL-17 protein in the supernatant from IL-17$^{-/-}$ T cell cultures were below the limit of detection by ELISA (Figure 2C).

IL-17$^{-/-}$ mice were generated from the cross between IL-17$^{+/-}$ mice at the expected Mendelian ratio. They were fertile and did not show any gross phenotypic abnormalities under specific pathogen-free housing conditions (data not shown). No apparent abnormalities were found in the cell populations of the thymus, LNs, and spleen among IL-17$^{+/+}$, IL-17$^{+/-}$, and IL-17$^{-/-}$ mice (data not shown).

T Cell Response to Mitogenic Stimuli

IL-17 is known as a T cell costimulatory factor (Yao et al., 1995a). To examine whether IL-17 is necessary for T cell activation, splenic T cells from IL-17$^{-/-}$ mice were stimulated with plate-coated anti-CD3 plus anti-CD28 mAb. Proliferative responses of IL-17$^{-/-}$ T cells and IL-4 and IFN-γ production were normal (Figures 2A and 2B), although IL-17 production was completely abolished (Figure 2C). Proliferative responses of whole spleen cells to other mitogenic stimuli such as ConA, PHA, and PMA plus ionomycin were also shown to be normal in IL-17$^{-/-}$ mice (Figure 2D). Therefore, these results indicate that IL-17 is not necessary for either cell proliferation or IL-4 or IFN-γ production by T cells in response to nonspecific mitogenic stimuli.

CHS

It was shown that nickel-specific skin-derived T cells produce IL-17, and this IL-17 induces IL-6 and IL-8 production as well as ICAM-1 and HLA-DR expression on

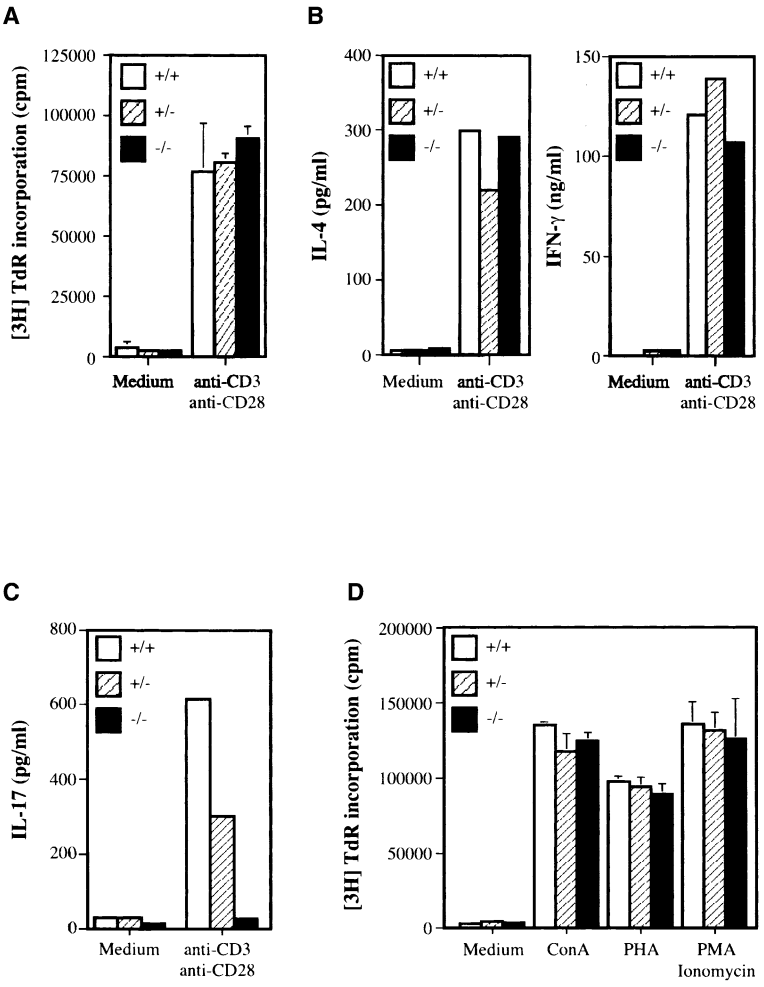
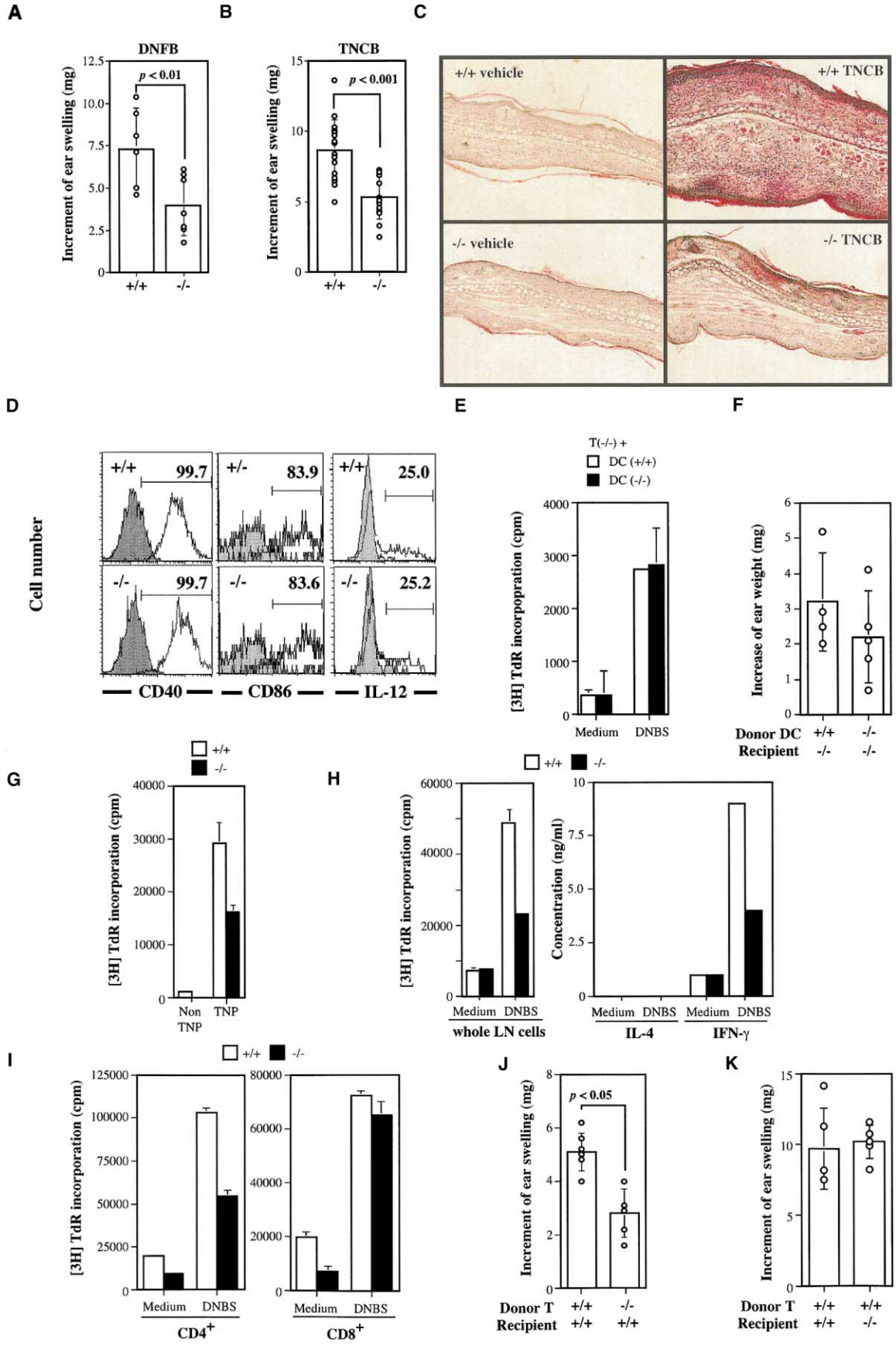


Figure 2. T Cell Functions of IL-17$^{-/-}$ Mice

Splenic T cells (A, B, and C) and whole spleen cells (D) from IL-17$^{+/+}$, IL-17$^{+/-}$, and IL-17$^{-/-}$ mice were stimulated with mitogenic stimuli. Proliferative responses (A), IL-4 and IFN-γ levels (B), and IL-17 levels (C) in the culture supernatants are shown after stimulation with plate-coated anti-CD3 plus anti-CD28 mAb for 48 hr, and proliferative responses to mitogens after 48 hr are shown in (D). Average ± SD of three wells is shown in the proliferation assay, and cytokine levels in pooled supernatant from three wells were determined by ELISA. These results were reproducible in two independent experiments.

keratinocytes in humans (Albanesi et al., 1999). To elucidate the role of IL-17 in hapten-specific skin diseases, we investigated CHS responses in IL-17$^{-/-}$ mice. DNFB- and TNCB-induced CHS responses in IL-17$^{-/-}$ mice were markedly suppressed compared with those in IL-17$^{+/+}$ mice (Figures 3A and 3B). The response in IL-17$^{+/-}$ mice was similar to that in IL-17$^{+/+}$ mice (data not shown). IL-17$^{+/+}$ mice exhibited infiltration of a large number of inflammatory cells into the TNCB-challenged ear epidermis, while cell infiltration was not shown in vehicle-treated ears (Figure 3C). In contrast, inflammatory cell infiltration in TNCB-treated ears was markedly reduced in IL-17$^{-/-}$ mice (Figure 3C).

IL-17 is known to enhance T cell activation via promotion of DC maturation (Antonysamy et al., 1999). Langerhans cells (LCs), which are the major antigen-presenting cells in CHS response, are immature in the skin and maturate during the migration process from the skin to draining LNs after activation with antigens (Grabbe and Schwarz, 1998). However, LC migrating into draining LNs from the skin expressed normally cell surface activation markers, CD40 and CD86, in IL-17$^{-/-}$ mice after painting with FITC on the skin (Figure 3D). Six hours after LPS stimulation, IL-12 expression in FITC^{+}CD11c^{+} LCs was also comparable between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice (Figure 3D). When T cells from DNFB-sensitized IL-17$^{-/-}$ mice were cocultured with CD11c^{+} DCs from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice in the absence or presence of DNBS, IL-17$^{-/-}$ DCs could promote T cell proliferation in a manner similar to IL-17$^{+/+}$ DCs (Figure 3E). Furthermore, when nontreated IL-17$^{-/-}$ mice were injected with TNP-conjugated splenic DCs from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice, the severity of CHS in mice that received IL-17$^{-/-}$ DCs was similar to that in IL-17$^{-/-}$ mice that received IL-17$^{+/+}$ DCs (Figure 3F). Thus, these results indicate that IL-17 deficiency does not affect LC/DC function during CHS response. On the other hand, TNP- and DNP-specific T cell proliferative responses from draining LNs of IL-17$^{-/-}$ mice were significantly decreased compared with those of IL-17$^{+/+}$ mice (Figures 3G and 3H). Among CD3^{+} cells, CD4^{+} T cells but not CD8^{+} T cells showed reduced proliferative response against DNBS (Figure 3I). IFN-γ production after stimulation with DNBS was markedly reduced in the IL-17$^{-/-}$ T cell culture, while IL-4 production was almost undetectable both in the IL-17$^{+/+}$ T cell and the IL-17$^{-/-}$ T cell cultures (Figure 3H). When TNCB-sensitized T cells were transferred into nontreated mice, the CHS response in mice that received T cells from IL-17$^{-/-}$ mice was significantly lower compared with the response in mice that received T cells from IL-17$^{+/+}$ mice (Figure 3J). However, when IL-17$^{-/-}$ mice were previously transplanted with CD4^{+} T


A
DNFB
p < 0.01
Increment of ear swelling (mg)
+/+
-/-
B
TNCB
p < 0.001
Increment of ear swelling (mg)
+/+
-/-
C
+/+ vehicle
+/+ TNCB
-/- vehicle
-/- TNCB
D
Cell number
+/+ 99.7
+/- 83.9
+/+ 25.0
-/- 99.7
-/- 83.6
-/- 25.2
CD40
CD86
IL-12
E
T(-/-) +
DC (+/+)
DC (-/-)
[3H] TdR incorporation (cpm)
Medium
DNBS
F
Increase of ear weight (mg)
Donor DC +/+ -/-
Recipient -/- -/-
G
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[3H] TdR incorporation (cpm)
Non TNP
TNP
H
+/+
-/-
[3H] TdR incorporation (cpm)
Medium DNBS
whole LN cells
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Medium DNBS Medium DNBS
IL-4
IFN-γ
I
+/+
-/-
[3H] TdR incorporation (cpm)
Medium DNBS
CD4+
Medium DNBS
CD8+
J
p < 0.05
Increment of ear swelling (mg)
Donor T +/+ -/-
Recipient +/+ +/+
K
Increment of ear swelling (mg)
Donor T +/+ +/+
Recipient +/+ -/-

cells from wild-type mice before sensitization, CHS in these reconstituted mice was recovered to the levels of wild-type mice (Figure 3K). Therefore, these results indicate that IL-17 is required for hapten-specific T cell activation, especially for $CD4^+$ T cells, but not for LC maturation in the sensitization phase of CHS response.

To elucidate the effects of IL-17 deficiency on humoral immunity, TNP-specific Ig levels in sera from IL-$17^{-/-}$ mice at 3 days after TNCB challenge were determined by ELISA. All TNP-specific IgG subclass levels in sera from IL-$17^{-/-}$ mice were significantly lower compared with those from IL-$17^{+/+}$ mice (Figure 4A). Thus, we showed that IL-17 also plays an important role in humoral immunity.

In the elicitation phase of CHS, various cells such as neutrophils, macrophages, and T cells were seen to have infiltrated the sites which were chemically challenged. In this phase, vascular permeability is important for cellular infiltration. Because of this, we investigated the vascular permeability of IL-$17^{-/-}$ mice, since IL-17 is thought to be involved in this process through the induction of *iNOS* and *cox-2* (Shalom-Barak et al., 1998). One hour after a TNCB challenge, exudation of Evans blue dye in the ear was normal in IL-$17^{-/-}$ mice (Figure 4B).

To assess the effect of IL-17 deficiency on the expression of proinflammatory cytokines, chemokines, and cell adhesion molecules, protein and mRNA levels were analyzed by ELISA and Northern blot hybridization analysis, respectively. IL-1α, IL-1β, and TNF-α levels in the lysate of the IL-$17^{-/-}$ mouse ears challenged with TNCB were similar to those of IL-$17^{+/+}$ mice. In addition, these cytokines were produced with similar kinetics (Figures 4C and 4D), although IL-17 was not produced in IL-$17^{-/-}$ mice (Figure 4C). On the other hand, the mRNA levels of chemokines were reduced by 20%–50% in IL-$17^{-/-}$ mice compared with IL-$17^{+/+}$ mice at 6 hr after TNCB challenge (Figure 4E). ICAM-1 expression also decreased partially in IL-$17^{-/-}$ mice (Figure 4E).

The level of neutrophil infiltration was estimated by MPO assay (Figure 4F). The results showed the involvement of IL-17 in this process, consistent with the results of a previous report (Fossiez et al., 1998) and with the finding that the expression of chemokine mRNAs for Groα, MIP-1, and MIP-2 were found to be reduced (Figure 4E). These results show that IL-17 also plays an important role in the infiltration process of inflammatory cells.

It is known that TNF-α is also involved in CHS response, and it was reported recently that IL-17 was expressed by T cells producing TNF-α (Infante-Duarte et al., 2000). We then examined whether or not the role of IL-17 in CHS response was different from that of TNF-α. TNF-α production in the inflammatory sites was normal in IL-$17^{-/-}$ mice, and IL-17 production on T cells of TNF-$\alpha^{-/-}$ mice was also found to be normal (data not shown). CHS responses in TNF-$\alpha^{-/-}$ mice as well as in IL-$17^{-/-}$ mice were similarly reduced compared with those in wild-type mice, and those in IL-$17^{-/-}$ x TNF-$\alpha^{-/-}$ mice were severely reduced compared with those in TNF-$\alpha^{-/-}$ or in IL-$17^{-/-}$ mice (Figure 4G).

These results suggest that the reduced CHS response in IL-$17^{-/-}$ mice is caused by a TNF-α-independent mechanism. Taken together, these results indicate that IL-17 has an important role in hapten-specific cellular and humoral immunities during CHS response.

DTH

We examined the role of IL-17 in the DTH response, which is a Th1 cell-mediated cellular immune response. Methylated-BSA (mBSA)-induced DTH response in IL-$17^{-/-}$ mice was suppressed to approximately 80% of the average response in IL-$17^{+/+}$ mice (Figure 5A). In IL-$17^{-/-}$ mice, cell infiltration at the inflammatory site was milder than in IL-$17^{+/+}$ mice (data not shown). The T cell proliferative response of IL-$17^{-/-}$ mice against mBSA was reduced compared with those of IL-$17^{+/+}$ mice (Figure 5B). Consistent with the proliferation levels, IFN-γ production in culture supernatants from IL-$17^{-/-}$ mice was also found to be lower than that from IL-$17^{+/+}$ mice (Figure 5C). IL-4 levels were below the level of detection

Figure 3. Impaired CHS Response in IL-$17^{-/-}$ Mice

(A) Increase of ear swelling in DNFB-induced CHS 24 hr after the second challenge.

(B) Increase of ear swelling in TNCB-induced CHS 24 hr after the second challenge.

(C) Histology of the ear skin at 24 hr after the challenge with TNCB or vehicle alone. Hematoxylin-eosin staining, $\times$40.

(D) CD40, CD86, and IL-12 expression on $CD11c^+$ $FITC^+$ LCs. The expression of activation marker on LCs migrating into draining LNs from the skin 24 hr after FITC painting was analyzed by FACS. For IL-12 expression, draining LNs from the skin 24 hr after painting with FITC were stimulated with 10 μg/ml LPS plus 2 μM monensin for 6 hr. Shaded area shows an isotype-matched control Ig staining.

(E) Antigen presentation ability of IL-$17^{-/-}$ DCs. DNFB-sensitized IL-$17^{-/-}$ T cells (5×10^5 cells) were cocultured with DCs from IL-$17^{+/+}$ or IL-$17^{-/-}$ mice in the absence or presence of 25 μg/ml DNBS for 3 days, and then [^{3}H] TdR incorporation was measured.

(F) CHS response after adoptive transfer of DCs. $CD11c^+$ splenic DCs were purified from IL-$17^{+/+}$ or IL-$17^{-/-}$ mice and treated with TNBS. Then, the TNP-conjugated DCs were transferred into IL-$17^{-/-}$ recipient mice, and CHS was measured after challenging with TNCB.

(G) Reduced TNP-specific T cell proliferation response in IL-$17^{-/-}$ mice. Inguinal and axillary LN T cells were purified from IL-$17^{+/+}$ or IL-$17^{-/-}$ mice sensitized with TNCB 5 days after the first challenge and were cocultured with TNBS-treated splenic adherent cells for 3 days.

(H) DNP-specific T cell proliferative responses and IL-4 and IFN-γ production. Inguinal and axillary LN cells from IL-$17^{+/+}$ or IL-$17^{-/-}$ mice sensitized with DNFB were harvested 5 days after the first challenge and cultured in the absence or presence of 50 μg/ml DNBS for 3 days.

(I) Inguinal and axillary LN $CD4^+$ or $CD8^+$ cells from IL-$17^{+/+}$ or IL-$17^{-/-}$ mice sensitized with DNFB were cocultured with splenic adherent cells in the absence or presence of 50 μg/ml DNBS for 3 days.

(J) CHS response after adoptive transfer of T cells. Inguinal and axillary LN T cells from TNCB-sensitized IL-$17^{+/+}$ or IL-$17^{-/-}$ mice were transferred, and CHS response of the T cell-transferred mice was examined by challenging TNCB on the ear.

(K) Reconstitution of IL-$17^{-/-}$ mice with IL-$17^{+/+}$ $CD4^+$ T cells. $CD4^+$ T cells (4×10^7 cells) were purified from IL-$17^{+/+}$ mice, and they were injected into naive IL-$17^{+/+}$ or IL-$17^{-/-}$ mice. Then, these mice were sensitized with TNCB, and CHS was measured.

(A, B, F, J, and K) Each circle represents an individual mouse, and average and SD are shown. (E, G, H, and I) Average $\pm$ SD of three wells is shown in the proliferation assay, and cytokine levels in a pooled supernatant from three wells from proliferative response assay were determined by ELISA. These results were reproducible in at least three independent experiments.

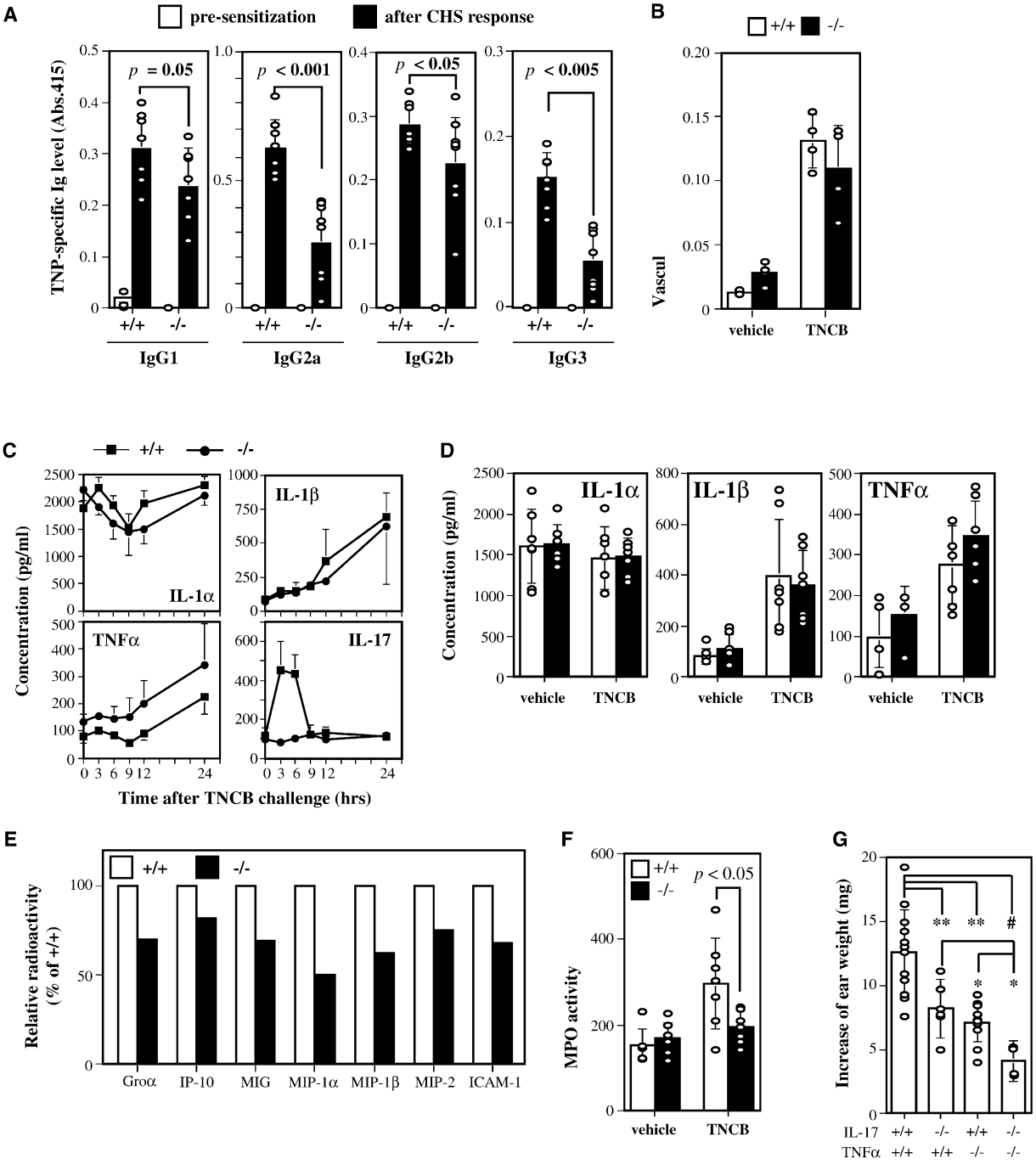


Figure 4. Impaired CHS Response in IL-17$^{-/-}$ Mice

(A) TNP-specific antibody production during CHS response. Three days after the challenge with TNCB, sera were collected and antibody levels were measured by ELISA.

(B) Vascular permeability in CHS response. Evans blue dye was injected intravenously after the challenge with TNCB, and 1 hr later, exudative dye in the ear skin was extracted, and the absorbancy at 650 nm was measured.

(C) IL-1α, IL-1β, TNF-α, and IL-17 levels in the ear after challenge with TNCB. The ears were removed at the time indicated after the second challenge, and cytokine levels in the lysate were measured by ELISA. The average of three mice is shown.

(D) IL-1α, IL-1β, and TNF-α levels in the lysate of the ear challenged with TNCB. Cytokine levels were measured by ELISA 24 hr after the challenge.

(E) Northern blot analysis of the mRNAs of chemokines and an adhesion molecule in the ear skin at 6 hr after the challenge with TNCB. β-actin mRNA was used to normalize the amount of loaded RNA, and the relative values in IL-17$^{-/-}$ mice compared to those in IL-17$^{+/+}$ mice are shown.

(F) MPO activity in CHS response. The lysate of the ear skin was prepared at 24 hr after the challenge with TNCB, and MPO activity was measured.

(G) TNCB-induced CHS responses in IL-17$^{-/-}$, TNF-α$^{-/-}$, and IL-17$^{-/-}$ x TNF-α $^{-/-}$ mice.

(A, B, D, F, and G) Each circle represents an individual mouse, and average and SD are shown. $p < 0.05$; **, $p < 0.01$; #, $p < 0.005$.

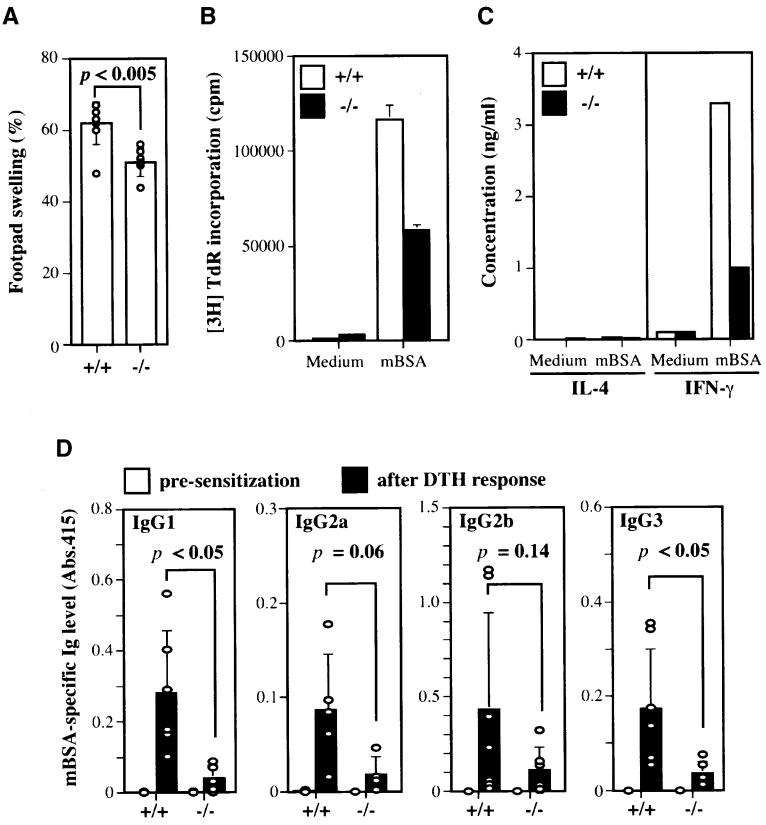


Figure 5. Impaired DTH Response in IL-17$^{-/-}$ Mice

(A) Increase of footpad thickness in mBSA-induced DTH response. Each circle represents an individual mouse, and average and SD are shown.

(B) mBSA-specific T cell proliferative response. Inguinal LN cells from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice sensitized with mBSA/CFA subcutaneously for 1 week were cultured in the absence or presence of 40 μg/ml mBSA for 3 days. The average ± SD of three wells is shown. These results were reproducible in three independent experiments.

(C) IL-4 and IFN-γ levels in the pooled supernatant of three wells from proliferative response assay were determined by ELISA. These results were reproducible in three independent experiments.

(D) mBSA-specific antibody levels after DTH reaction. One week after the challenge with mBSA, sera were collected, and mBSA-specific antibody levels were measured. Each circle represents an individual mouse, and average and SD are shown.

in both IL-17$^{+/+}$ and IL-17$^{-/-}$ culture supernatants (Figure 5C). One week after the DTH response, mBSA-specific IgG1 and IgG3 levels in sera from IL-17$^{-/-}$ mice were significantly reduced compared with those in IL-17$^{+/+}$ mice (Figure 5D). These results indicate that IL-17 also plays an important role in the induction of the Th1-mediated DTH response.

AHR

IL-17 is found in the sera of allergic asthma patients (Wong et al., 2001). In order to assess whether or not IL-17 is involved in the development of asthma, we analyzed OVA/alum-induced AHR using IL-17$^{-/-}$ mice. Five days after the first immunization with OVA/alum intraperitoneally, the proliferative response of the mesenteric LN cells from IL-17$^{-/-}$ mice against OVA was markedly reduced compared with that of the IL-17$^{+/+}$ mice (Figure 6A). IL-4 and IL-5 levels in the supernatant of IL-17$^{-/-}$ LN cell cultures were also lower than those of IL-17$^{+/+}$ LN cell cultures, while IFN-γ levels of both IL-17$^{+/+}$ and IL-17$^{-/-}$ LN cell cultures were similar (Figure 6B).

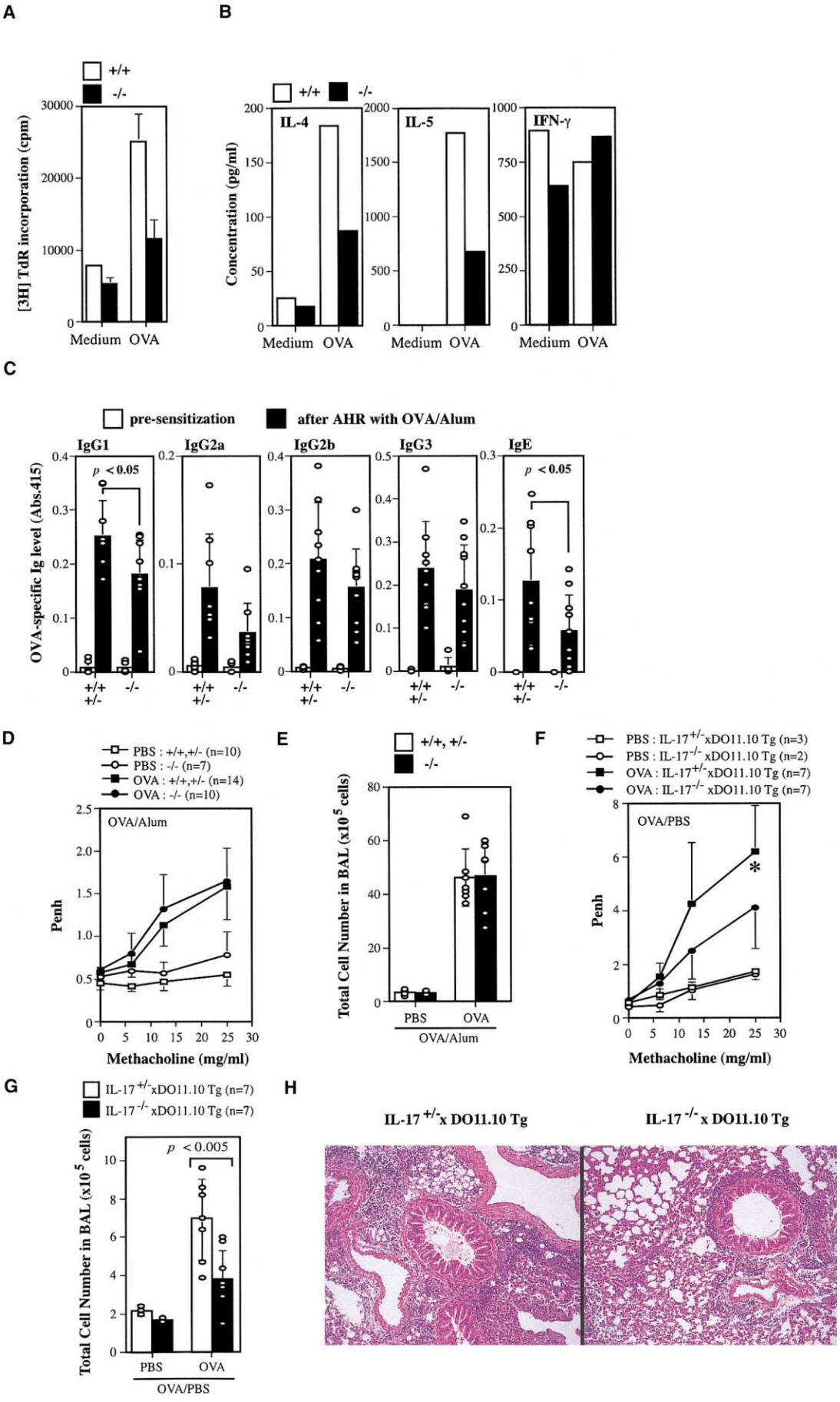
Furthermore, when mice were immunized with OVA/alum intraperitoneally, OVA-specific IgG1 and IgE levels in IL-17$^{-/-}$ mice were significantly reduced compared with those in IL-17$^{+/+}$ mice (Figure 6C). In spite of the reduced OVA-specific T cell responses, the AHR to methacholine in IL-17$^{-/-}$ mice were similar to those of IL-17$^{+/+}$ and IL-17$^{+/-}$ mice (Figure 6D).

The total infiltrated cell numbers in bronchoalveolar lavage fluids (BALF) (Figure 6E) and the pulmonary pathology (data not shown) were not different between the IL-17$^{+/+}$, IL-17$^{+/-}$, and IL-17$^{-/-}$ mice. Moreover, IL-4 and IL-5 levels in the BALF from IL-17$^{-/-}$ mice at 24 hr after the last inhalation with OVA were higher than those from IL-17$^{+/+}$ and IL-17$^{+/-}$ mice (data not shown). The apparent discrepancy between the reduced T cell activation and normal AHR with pulmonary inflammation in IL-17$^{-/-}$ mice may be explained by the effect of alum, which induces an excess Th2 response independently from IL-4 and IL-13 signaling (Brewer et al., 1999).

We then examined the role of IL-17 in OVA-induced AHR without alum using IL-17$^{-/-}$ x DO11.10 transgenic (Tg) mice. Twenty-four hours after the last inhalation with OVA, the AHR to methacholine in IL-17$^{-/-}$ x DO11.10 Tg mice was partially decreased compared with that in IL-17$^{+/-}$ x DO11.10 Tg mice (Figure 6F). Consistent with AHR, the number of total infiltrated cells in BALF from IL-17$^{-/-}$ x DO11.10 Tg mice was reduced compared with that of IL-17$^{+/-}$ x DO11.10 Tg mice (Figure 6G). As expected, pulmonary inflammation in IL-17$^{-/-}$ x DO11.10 Tg mice was also found to be milder than that of IL-17$^{+/-}$ x DO11.10 Tg mice (Figure 6H). These observations indicate that IL-17 is involved in the induction of AHR.

Acute GVHR

We then examined the role of IL-17 in acute GVHR. When splenocytes from IL-17$^{-/-}$ or IL-17$^{+/+}$ mice on the C57BL/6J background (H-2K^{b}) were transferred into CBF1 mice (H-2K$^{b/d}$), the number of IL-17$^{-/-}$ CD4^{+} T cells in the spleen of recipient CBF1 mice was similar to that of IL-17$^{+/+}$ CD4^{+} T cells (Figure 7A). A similar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IL-4
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Concentration (pg/ml)
Medium OVA
C
pre-sensitization
after AHR with OVA/Alum
IgG1
IgG2a
IgG2b
IgG3
IgE
p < 0.05
OVA-specific Ig level (Abs.415)
+/+ +/-
-/-
D
PBS : +/+,+/- (n=10)
PBS : -/- (n=7)
OVA : +/+,+/- (n=14)
OVA : -/- (n=10)
OVA/Alum
Penh
Methacholine (mg/ml)
E
+/+, +/-
-/-
Total Cell Number in BAL (x10^5 cells)
PBS OVA
OVA/Alum
F
PBS : IL-17+/- xDO11.10 Tg (n=3)
PBS : IL-17-/- xDO11.10 Tg (n=2)
OVA : IL-17+/- xDO11.10 Tg (n=7)
OVA : IL-17-/- xDO11.10 Tg (n=7)
OVA/PBS
Penh
Methacholine (mg/ml)
G
IL-17+/- xDO11.10 Tg (n=7)
IL-17-/- xDO11.10 Tg (n=7)
p < 0.005
Total Cell Number in BAL (x10^5 cells)
PBS OVA
OVA/PBS
H
IL-17+/- x DO11.10 Tg
IL-17-/- x DO11.10 Tg

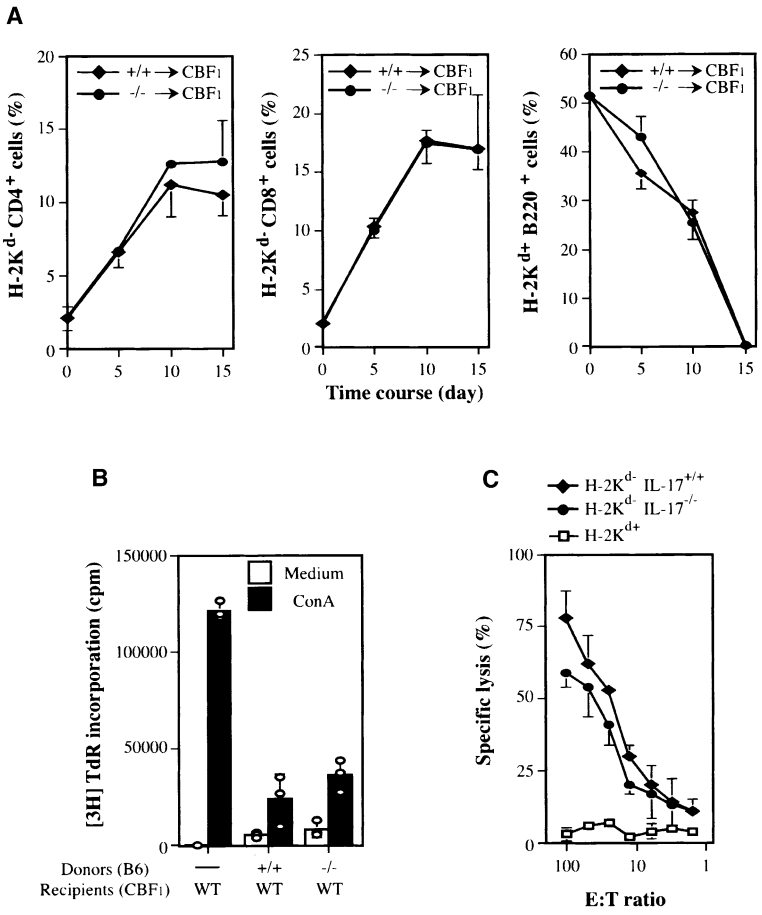

Figure 7. Normal Acute GVHR by IL-17$^{-/-}$ Donor Cells

Spleen cells from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice on the C57BL/6J background (H-2K$^{b/b}$) were transferred into CBF1 (C57BL/6J x BALB/cA F1: H-2K$^{b/d}$) mice intravenously.

(A) At indicated time points after the transfer, the spleen of the recipient CBF1 mice was removed, and the expansion of H-2K^{d-}CD4$^+$ or H-2K^{d-}CD8$^+$ cells of the donor IL-17$^{+/+}$ or IL-17$^{-/-}$ spleen cells and the reduction of H-2K^{d+}B220$^+$ cells of the recipient CBF1 mice were analyzed by FACS. Symbols represent the average of three mice and SD.

(B) Proliferative responses of the recipient CBF1 spleen cells to ConA at 10 days after transfer. Each circle represents an individual mouse, and average and SD are shown.

(C) CTL activity to P815 cells (H-2K^{d+}). Ten days after the transfer, H-2K^{d-}CD8$^+$ T cells were purified from the recipient CBF1 spleen cells by the MACS system. Specific lysis of H-2K^{d+}P815 cells by H-2K^{d-}CD8$^+$ T cells was determined by the ^{51}Cr release assay. Symbols represent the average of three mice and SD.

tendency was also observed in CD8$^+$ T cells from IL-17$^{-/-}$ mice (Figure 7A). The reduction kinetics of the recipient B cells was also similar in mice that received IL-17$^{+/+}$ and IL-17$^{-/-}$ T cells (Figure 7A). The ConA response of the recipient spleen cells from CBF1 mice that received IL-17$^{+/+}$ and IL-17$^{-/-}$ spleen cells was similarly reduced (Figure 7B). No difference was observed between IL-17$^{+/+}$ and IL-17$^{-/-}$ CD8$^+$ T cells in the cytotoxic T cell activity to H-2^d-specific P815 target cells (Figure 7C). These results indicate that IL-17 deficiency of donor T cells does not affect the development of acute GVHR.

Discussion

In the current study, we first generated IL-17$^{-/-}$ mice and demonstrated that IL-17 is involved in the development of various allergic diseases by activating allergen-

Figure 6. AHR in IL-17$^{-/-}$ Mice

(A) OVA-specific T cell proliferative response. Mesenteric LN cells from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice sensitized with OVA/alum intraperitoneally were cultured in the absence or presence of 40 μg/ml OVA for 3 days. The average ± SD of triplicate experiments is shown. These results were reproducible in three independent experiments.

(B) IL-4, IL-5, and IFN-γ levels in pooled supernatant from three wells from proliferative response assay were determined by ELISA. These results were reproducible in three independent experiments.

(C) OVA-specific antibody levels after AHR. Twenty-four hours after the last challenge with OVA, sera were collected, and OVA-specific Ig levels were determined by ELISA. Each circle represents an individual mouse, and average and SD are shown.

(D) AHR to aerosolized methacholine in mice sensitized with OVA/alum intraperitoneally and challenged with OVA/PBS or PBS intranasally. Airway bronchoconstriction was assessed by enhanced respiratory pause (Penh).

(E) Total cell numbers in BALF. At 24 hr after the last inhalation of OVA/PBS or PBS in mice sensitized with OVA/alum, the trachea was cannulated and the airways were lavaged three times with 1 ml of PBS. The BALF was centrifuged, and cell numbers were counted manually.

(F) AHR in IL-17$^{-/-}$ x DO11.10 Tg mice, who were treated with OVA/PBS or PBS intranasally without prior sensation to OVA. Airway bronchoconstriction was assessed by Penh.

(G) Total cell numbers in BALF. At 24 hr after the last inhalation of OVA/PBS or PBS in IL-17$^{+/-}$ or IL-17$^{-/-}$ x DO11.10 Tg mice, BALF was collected and cell numbers were counted manually as in (E). Each circle represents an individual mouse, and average and SD are shown.

(H) Lung histology in IL-17$^{+/-}$ or IL-17$^{-/-}$ x DO11.10 Tg mice inhaled with OVA/PBS. At 24 hr after the last inhalation, the lungs were removed and sections of lung were stained with hematoxylin-eosin (×60). Symbols represent the average of each genotype mice with SD in (D) and (F). Each circle represents an individual mouse, and average and SD are shown in (C), (E), and (G). *, $p < 0.05$.

specific T cells. We have shown that the CHS response was weak in IL-17$^{-/-}$ mice, indicating involvement of IL-17 in this response.

Production of IL-17 is also reported in human skin-derived nickel-specific T cells (Albanesi et al., 2000). The CHS response is induced via multiple steps. At the initial phase, LCs in the skin migrate into LNs upon stimulation with contact allergens; then, LCs maturate in LNs and activate allergen-specific T cells through T-LC interaction in the sensitization phase; and in the elicitation phase, these allergen-primed T cells infiltrate into the skin and induce inflammation upon rechallenging with the allergens (Grabbe and Schwarz, 1998). We showed that IL-17 deficiency affected neither migration (data not shown) nor maturation of LCs (Figure 3D), although it was reported that exogenously added IL-17 promoted maturation of DCs (Antonysamy et al., 1999). Instead, we found that IL-17 is required for the activation of hapten-specific CD4$^+$ but not CD8$^+$ T cells. Adoptive transfer experiments of TNCB-immunized T cells clearly indicated that IL-17 is required for the sensitization phase.

These observations suggest that the reduced CHS response in IL-17$^{-/-}$ mice is caused by an insufficient hapten-specific CD4$^+$ T cell activation in the sensitization phase. With regard to this, it was shown previously using MHC class I$^{-/-}$ and MHC class II$^{-/-}$ mice that the effector cells in CHS response are CD8$^+$ T cells and their activity is regulated by CD4$^+$ T cells (Bouloc et al., 1998). Recently, however, it was shown using CD8$^{-/-}$ and CD4$^{-/-}$ mice that both CD8$^+$ and CD4$^+$ T cells are involved in the development of CHS, indicating that CD4$^+$ T cells are necessary for full CD8$^+$ T cell activation (Wong et al., 2000).

Proinflammatory cytokines such as IL-1 and TNF-α are known to have crucial roles in the induction of other cytokines, chemokines, and adhesion molecules that play important roles in various inflammatory responses. It has been suggested that IL-17 has activities similar to these cytokines and is also known to be a potent inducer of IL-1 and TNF-α acting on macrophages and kerationocytes (Albanesi et al., 1999, 2000; Jovanovic et al., 1998).

Although the functional discrimination and synergy among these cytokines are not completely known, there are some indications that suggest a unique role for each cytokine in the CHS reaction. In the elicitation phase, IL-1α, IL-1β, and TNF-α were produced normally at the inflammatory sites in IL-17$^{-/-}$ mice both in terms of the induction kinetics and the protein levels, indicating that the defect of the CHS reaction was directly caused by the IL-17 deficiency and not by a deficiency of other cytokines.

We showed previously that IL-1 produced by APCs is required for hapten-specific T cell activation in the sensitization phase and induces CD40L and OX40 on T cells (Nakae et al., 2001a, 2001c). At present, we do not know the molecular mechanisms for the activation of T cells by IL-17. We only know that IL-17 production by hapten-specific T cells is reduced in IL-1$\alpha/\beta^{-/-}$ mice (Nakae et al., submitted). We are now analyzing the possible mechanisms.

We found that, both IL-1 and TNF-α, but not IL-17, were involved in vascular permeability at the inflammatory sites after challenge with TNCB (Figure 4B and our unpublished data). Furthermore, we showed that TNF-α was not necessary for the sensitization phase because mice that received T cells from TNF-$\alpha^{-/-}$ mice developed a normal CHS response (Nakae et al., submitted). This finding is in clear contrast to the effect of IL-17 deficiency, in which CHS response was significantly suppressed in IL-17$^{-/-}$ T cell-transplanted mice. These observations indicate that IL-17 and TNF-α play roles in different processes. Consistent with this notion, we found that CHS response in IL-17$^{-/-}$ x TNF-$\alpha^{-/-}$ mice was significantly lower than in single cytokine knockout mice as shown in Figure 4G.

AHR is a typical Th2-dependent immune reaction, and IL-4, IL-5, and IL-13 are believed to be the key molecules involved in the production of IgE and for the activation of mast cells and eosinophils (Lloyd et al., 2001). OVA/alum-induced AHR developed normally in IL-17$^{-/-}$ mice, although IL-4 and IL-5 production by T cells and antibody production to OVA were reduced. In contrast, AHR induced by OVA/PBS inhalation was suppressed in IL-17$^{-/-}$ x DO11.10 Tg mice. The fact that alum can induce an excess Th2 response without IL-4Rα signaling (Brewer et al., 1999) may be the cause of the difference seen between OVA/alum and OVA/PBS.

It is known that many asthmatic patients can develop dramatic pathophysiological responses to challenge with low doses of allergens (Sulakvelidze et al., 1998). This indicates that AHR model induced by OVA/PBS, rather than that by OVA/alum, seems to reflect more closely the pathology in humans. This notion is supported by the observation that B cell-deficient mice, as well as mast cell-deficient mice, exhibited a normal AHR when the animals were sensitized with OVA/alum (Korsgren et al., 1997; Takeda et al., 1997), although the generally accepted concept is that activation of mast cells by the antigen/IgE complex is crucial for the development of AHR (Martin et al., 1993). On the other hand, AHR in mast cell-deficient mice sensitized with OVA without alum was markedly suppressed (Williams and Galli, 2000). Moreover, we observed a similar phenomenon when AHR was induced with OVA in IL-1$\alpha/\beta^{-/-}$ mice: AHR was suppressed only when mice were immunized with OVA/PBS but not with OVA/alum (our unpublished data). Although T cell functions such as proliferation against OVA and cytokine production were suppressed in IL-17$^{-/-}$ mice sensitized with OVA/alum, these low levels of T cell function seem to be enough to induce full AHR. These findings indicate that IL-17 is not essential for the development of allergic airway hypersensitivity under strong AHR-inducing conditions using adjuvant, but rather that IL-17 plays an important role under conditions of mild inducement through activation of allergen-specific T cells.

DTH response is a Th1 cell-mediated cellular immune response (Grabbe and Schwarz, 1998). We found that the DTH response was also impaired in IL-17$^{-/-}$ mice in the same way as in Th2-mediated AHR or Th1- and Tc1-mediated CHS, and that IL-17 was required for allergen-specific T cell activation in the sensitization phase during DTH, similarly to other responses. Thus, IL-17 seems to play an important role in activation of both Th1 and Th2 cells.

Host-versus-graft reaction (HVGR) is another T cell-

mediated immune response. Blockade of IL-17 binding with IL-17R-Fc, an IL-17R extracellular domain fused with Fc fragment, caused prolonged allograft survival (Antonysamy et al., 1999), which suggests the involvement of IL-17 in HVGR. In GVHR, however, we showed that IL-17 deficiency of donor T cells did not affect the survival of recipient B cells and that alloantigen-specific CTL activity of IL-17$^{-/-}$ CD8$^+$ T cells was normal. These observations indicate that donor CD4$^+$ T cell-produced IL-17 is not required for allo-specific CD8$^+$ T cell activation in acute GVHR. Consistent with this, it was reported that expansion of allo-specific CD8$^+$ T cells occurred in acute GVHR without the help of CD4$^+$ T cells, the major producer of IL-17 (Buhlmann et al., 1999).

We showed that antigen-specific antibody production was significantly decreased in IL-17$^{-/-}$ mice. This effect of IL-17 deficiency was not affected by the route of immunization or the presence of adjuvant. The suppression of antibody production was observed in the CHS response, in which mice were sensitized with allergens via the skin route without adjuvants; in the DTH response, in which mice were immunized intraperitoneally with CFA; and in AHR, in which immunization was carried out intraperitoneally with alum. Since IL-17 deficiency did not have any direct effects on B cells as assessed by the response against mitogenic stimuli such as LPS, anti-IgM mAb, and anti-CD40 mAb (data not shown), these findings suggest that the observed defects on antibody production are due to insufficient B cell activation by IL-17$^{-/-}$ T cells.

Taken together, this study shows that IL-17 plays a crucial role in allergen-specific cellular and humoral immune responses through the activation of T cells, and we have also provided insights into molecular mechanisms for the development of CHS, DTH and AHR. It should be noted that IL-17 is specific because it mediates both Th1-dominant allergic reactions such as CHS, DTH, and collagen-induced arthritis (our unpublished data), and Th2-dominant reactions such as AHR. It is reported that IL-17 is produced by a specific T cell subpopulation that does not belong to Th1 or Th2 subpopulations and produces TNF-α and/or GM-CSF simultaneously (Infante-Duarte et al., 2000). Thus, these observations indicate that IL-17 belongs to a distinct category of cytokine that belongs to neither Th1 nor Th2 cytokines. These findings should be useful for the development of therapeutics for these diseases. Using these IL-17$^{-/-}$ mice, we are now examining the possibility that IL-17 is also involved in the development of other T cell-mediated autoimmune diseases such as collagen-induced arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis.

Experimental Procedures

Generation of IL-17$^{-/-}$ Mice

Genomic DNA containing the *il-17* gene was isolated from mouse 129/SVJ genomic phage library (Stratagene, La Jolla, CA). A targeting vector was constructed by replacing 2.1 kb genomic fragment with the 2.5 kb DNA fragment containing the *EGFP* gene and *neomycin resistance* gene (*neo*) under the control of the phosphoglycerate kinase (PGK) 1 promoter which was flanked by lox P sequences. The fragment contained the first and second exons, which ranged from the initiation codon ATG to a SnaBI site between the second and third exons. A *diphtheria toxin A* (*DT*) gene under the MC1 promoter was ligated to the 5′ end of the targeting vector for negative selection. The targeting vector was electroporated into ES (E14.1) cells and selected in G418 (Asano et al., 1997). Targeted clones were screened by Southern blot hybridization analysis using 5′ and 3′ probes. Two clones out of seven independently identified targeted ES clones were treated with Adeno virus carrying the *cre* gene to delete the *neo* gene (Kanegae et al., 1995). Chimera mice were generated by the aggregation method using C57BL/6J blastocysts as the recipients (Asano et al., 1997). Chimera mice were mated with C57BL/6J female mice for germline transmission. For AHR, IL-17$^{-/-}$ mice were backcrossed to BALB/cA mice for three generations, and then crossed to DO11.10 transgenic mice, which were kindly provided by Dr. Dennis Y. Loh (Washington University School of Medicine). For CHS and DTH, littermates of IL-17$^{-/-}$ mice on 129 x B6 F1 background were used. The genotyping of IL-17$^{-/-}$ mice was carried out using the following PCR primers: primer 1, 5′-ACTCTTCATCCACCTCACACGA-3′; primer 2, 5′-GCCATGATATA GACGTTGTGGC-3′; primer 3, 5′-CAGCATCAGAGACTAGAAGGGA-3′. Primers 1 and 2 were used to detect wild-type allele (1.3 kb), and primers 1 and 3 were used to detect mutant allele (0.5 kb). TNF-$\alpha^{-/-}$ mice were backcrossed to C57BL/6J mice for ten generations and used for the experiments.

All the mice were kept under specific pathogen-free conditions in an environmentally controlled clean room in the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

Cell Proliferation Assay

Cell preparation and mitogenic response were performed as described previously (Nakae et al., 2001a, 2001b). To measure the mBSA- or OVA-specific LN cell response, LN cells (4×10^5 cells/well) were cultured in the absence or presence of 40 μg/ml of mBSA or OVA for 3 days, respectively, followed by incorporation of [^{3}H] thymidine (0.25 μCi/ml) (Amersham, Buckinghamshire, England) for 6 hr. Data shown are from the time point of the maximal response.

ELISA for Cytokines and Antigen-Specific Igs

For cytokine ELISA, monoclonal rat anti-mouse IL-17 (DAKO, Carpinteria, CA), hamster anti-mouse IL-1α mAb (Genzyme, Cambridge, MA), hamster anti-mouse IL-1β mAb (Genzyme), and anti-mouse TNF-α mAb (ENDOGEN, Woburn, MA) as capture Abs and polyclonal biotinylated goat anti-mouse IL-17 antibody (DAKO), rabbit anti-mouse IL-1α (Genzyme), rabbit anti-mouse IL-1β (Genzyme), and rabbit anti-mouse TNF-α (provided by Dr. Katsuo Noguchi, Teikyo University) as the second antibodies were used. HRP-avidin and HRP-conjugated goat anti-rabbit IgG was purchased from PharMingen and Zymed (San Francisco, CA), respectively. TMB substrate was purchased from DAKO. ELISA for IL-4, IL-5, and IFN-γ was performed as previously described (Nakae et al., 2001b). TNP-, mBSA-, and OVA-specific Ig levels were measured by ELISA as previously described (Nakae et al., 2001a, 2001c; Ohshima et al., 1998).

CHS

TNCB-induced CHS, TNP-specific T cell proliferation, LC preparation and maturation, and intracellular staining of FITC$^+$CD11c$^+$ LCs were assayed as described previously (Nakae et al., 2001c). In brief, abdomen of mice were sensitized epicutaneously with TNCB; then, 5 days later, the ear was challenged with 1% TNCB. At 24 hr after the second challenge, mice were euthanized, and discs of ear tissue were removed and weighed. Ear swelling was calculated as follows; (Increment of ear swelling [mg]) = {(weight of challenged ear [mg]) − (weight of vehicle-treated ear [mg])}. For FACS staining, PE-anti-mouse CD40 mAb (3.23; Immunotech, Marseille Cedex, France), PE-anti-mouse CD86 mAb (RMMP-1; Immunotech), and PE anti-IL-12 p40/p70 (C15.6: kindly provided by Dr. T. Tamura) were used. For DC transfer, TNP-conjugated CD11c$^+$ splenic DCs (5×10^5 cells) were transferred into nontreated mice, and then DC-transferred mice were challenged with TNCB. DNFB-induced CHS, DNP-specific T cell response, and adoptive T cell transfer were performed according to Chen et al. (1999). For reconstitution assay, CD4$^+$ T

cells (4×10^7 cells) from IL-17$^{+/+}$ mice were injected into nontreated IL-17$^{+/+}$ or IL-17$^{-/-}$ mice, and the next day, these mice were sensitized and challenged with TNCB. Vascular permeability was examined with a modification of the method described previously (Rauschmayr et al., 1997). At 5 min after the challenge with 1.0% TNCB, 8.3 μl/g body weight of 1.0% Evans blue dye (WAKO, Osaka, Japan) in PBS was injected through the tail vain. One hour later, a 6 mm diameter biopsy was taken from a TNCB- and/or vehicle-treated ear, Evans blue dye in the tissues was extracted in acetone, and the absorbancy at 650 nm was measured. A 6 mm diameter biopsy was taken from the TNCB- or vehicle-challenged ear, and after homogenization, MPO activity was measured as previously described (Higgins et al., 1999).

DTH

mBSA-induced DTH was examined as previously described (Zheng et al., 1995). In brief, mice were sensitized with 1.25 mg/ml mBSA (SIGMA, St. Louis, MO) with complete Freund's adjuvant (CFA; Difco, Detroit, MI) subcutaneously at the base of the tail. Seven days after sensitization, mice were challenged with 200 μg/20 μl mBSA into one footpad, and an equal volume of PBS was injected into another footpad. The footpad swelling was measured by a dial caliper and calculated as follows: (footpad swelling [%]) = {(footpad thickness of mBSA-injected footpad [mm]) – (footpad thickness of PBS-injected footpad [mm])} ÷ (footpad thickness of PBS-injected footpad [mm]) × 100.

AHR

Mice were sensitized with 100 μg/ml OVA/alum intraperitoneally on days 0 and 12, and 21 days later mice were challenged with 100 ng of OVA/PBS intranasally once a day for 3 days. For OVA/PBS-induced AHR, IL-17$^{+/-}$ or IL-17$^{-/-}$ mice crossed with DO11.10 Tg mice were made to inhale 100 ng of OVA/PBS intranasally every day for 4 days. Twenty-four hours after the last inhalation, AHR to methacholine was assessed using the parameter Penh (enhanced pause), which is calculated automatically based on the mean pressure generated in plethysmograph chambers during inspiration and expiration with the Buxco system as described elsewhere (Williams and Galli, 2000).

Acute GVHR

Acute GVHR was examined as previously described (Buhlmann et al., 1999). Splenocytes (6×10^7 cells) of IL-17$^{+/+}$ or IL-17$^{-/-}$ mice backcrossed to C57BL/6J mice for four generations were injected into a (C57BL/6 × BALB/c)F1 (CBF1) mouse intravenously. Anti-mouse CD16/CD32 (2.4G2), PE anti-mouse CD4 (RM4-5), PE anti-mouse CD8 (53-6.72), PE anti-mouse CD45R/B220 (RA3-6B2), and FITC anti-mouse H-2K^d (SF1-1.1) for FACS analysis were obtained from PharMingen. Spleen cell proliferation was examined 10 days after the transfer. After incubation with biotinylated anti-mouse H-2K^d and streptavidin-beads, H-2K^{d-}CD8$^+$ T cells were purified using MACS columns and used as the effector cells for CTL assay. P815 cells were used as target cells, and ^{51}Cr release was measured according to a standard protocol.

Statistics

Student's t test was used for statistical evaluation of the results.

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