CHAPTER IV

TNFα Induced by IL-1 Is Required for IP-10 Induction Independent of IFN-γ Signaling in the Elicitation Phase of Contact Hypersensitivity Response

Summary

Interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) are known as proinflammatory cytokines and have overlapping biological activities in various inflammatory responses. To understand specific and/or redundant roles between IL-1 and TNFα, I induced contact hypersensitivity response (CHS) using IL-1α/$\beta^{-/-}$, TNF$\alpha^{-/-}$ and IL-1α/$\beta^{-/-}$xTNF$\alpha^{-/-}$ mice. CHS in IL-1α/$\beta^{-/-}$ and TNF$\alpha^{-/-}$ mice was similarly reduced compared with that in wild-type mice, however, IL-1- or TNFα-deficiency had different effects on the induction of CHS. IL-1, but not TNFα, was required for hapten-specific T cell-priming in the sensitization phase, whereas TNFα induced by IL-1 was necessary for induction of local inflammation in the elicitation phase by inducing interferon-γ (IFN-γ)-inducible protein 10 (IP-10) not through IFN-γ signaling. IP-10 mRNA expression during CHS was abrogated in TNF$\alpha^{-/-}$ mice while CHS and TNFα mRNA expression in IFN-$\gamma^{-/-}$ mice exhibited normally. Interestingly, CHS in wild-type mice treated with anti-IP-10 neutralizing Ab was suppressed, suggesting that IFN-γ-independent and TNFα-dependent IP-10 plays an important role in CHS. Indeed, the reduced CHS in TNF$\alpha^{-/-}$ mice was recovered by IP-10 injection in the elicitation phase. Therefore, IL-1 and TNFα have distinct activities in the sensitization and elicitation phases of CHS, and IL-1-induced TNFα plays a crucial role in IP-10 induction independent of IFNγ signaling in the elicitation phase of CHS.

Introduction

Contact hypersensitivity response (CHS) consists of various events including migration and maturation of Langerhans cells (LC), T cell activation and leukocyte infiltration [1]. Various cytokines such as IL-1 and TNFα and chemokines produced by keratinocytes or LC were shown to involve in these events [2]. IL-1 and TNFα are known to promote the migration of LC [3-9] by regulating the expression of E-cadherin on keratinocytes and LC [10,11], and CCR7 on immature dendritic cells such as LC [12,13]. TNFα also involves in the expression of secondary lymphoid organ chemokine (SLC) and EBV-induced molecule 1 ligand chemokine (ELC), which are ligands for CCR7 [14]. IL-1 potentiates T-cell priming independently of CD28-CD80/CD86 co-signaling, especially IL-1α produced by LC migrating into LNs plays an important role in T cell activation in a sensitization phase of CHS [9,15]. Expression of adhesion molecules on vascular endothelial cells is also up-regulated by IL-1 and TNFα in an elicitation phase of CHS [16,17]. Thus, involvement of IL-1 and TNFα in the various steps of the development of CHS has been well documented. Indeed, it was demonstrated that CHS was suppressed in IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice, respectively [9,18]. However, CHS in IL-1α/β$^{-/-}$ mice as well as in TNFα$^{-/-}$ mice could not be suppressed completely, suggesting that the activities of TNFα in IL-1α/β$^{-/-}$ mice and those of IL-1α/β in TNFα$^{-/-}$ mice compensate their deficient function mutually. However, details of the specific and/or redundant roles between IL-1 and TNFα in the sensitization and elicitation phases during CHS still remain to be clarified.

Thus, the current study is carried out to determine the distinct and/or overlapping functions of IL-1 and TNFα in CHS using IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$ x TNFα$^{-/-}$ mice. My present results showed that IL-1 and TNFα play important roles in both sensitization and elicitation phases of CHS. In the sensitization phase, although these two cytokines were shown to be important for the migration of LC from the skin to LNs, IL-1 was required for hapten-specific T cell priming but TNFα was not. In the elicitation phase, IL-1 was shown to induce TNFα mRNA expression, and TNFα played a crucial role in the elicitation phase through induction of IP-10 expression.

Materials and Methods

Mice

IL-1α^{-/-}, IL-1β^{-/-}, IL-1α/β[-/-] and IFN-γ^{-/-} mice were generated by homologous recombination as described previously and backcrossed to C57BL/6J and BALB/cA mice for 8 generations, respectively [43,44]. TNFα^{-/-} mice were generated by Dr. K. Sekikawa and backcrossed to C57BL/6J and BALB/cA mice for 10 and 8 generations, respectively [45]. IL-1α/β[-/-]xTNFα^{-/-} mice were obtained by intercrossing IL-1α/β[-/-] and TNFα^{-/-} mice. TNFRI^{-/-} mice on C57BL/6J background (8 generetions) were originally generated by Dr. T. Mak. TNFRII^{-/-} mice on C57BL/6 background were obtained from Jackson Laboratory. These mice were housed under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guideline for gene manipulation experiments. Sex- and age-matched mice of 8 to 12 week-old were used throughout present experiments.

CHS

TNCB (Tokyo Kasei, Tokyo, Japan)-induced CHS was assayed as described previously (Zheng et al., 1995; Shornick et al., 1996). Briefly, abdomen of mice was shaved and sensitized epicutaneously with 25 μl of 3.0% TNCB dissolved in acetone mixed with olive oil mixture (4:1). On day 5 after the sensitization, the outside of one ear (auricle) was challenged with 25 μl of 1.0% TNCB and the outside of another ear was applied with 25 μl of vehicle alone. 24 h after the TNCB challenge, mice were euthanized and removed a disc of ear tissue from both ears of each mouse using a 6 mm biopsy punch. Each disc was weighed, and ear swelling is calculated as follows; [Increase of ear swelling] = ([weight of TNCB challenged ear] - [weight of vehicle-treated ear]). For reconstitution or neutralization of CHS with recombinant protein or Ab, 25 ng of mouse rTNFα (Peprotech), 50 ng of mouse rIL-1α/β mixture (Peprotech), 1 μg of mouse rIP-10 (Peprotech) or anti-mouse IP-10 mAb (provided by Dr. S. Narumi) was injected intradermally into ear skin immediately after challenge with TNCB. At 24 h after TNCB-challenge, ear swelling was measured by described above.

Migration and maturation of LCs

Back and abdomen of mouse were shaved and painted with 50 μl of 0.5% FITC isomer I (SIGMA) dissolved in a mixture of equal volumes of acetone and dibutylphthalate.

24 h later, inguinal, axillary and brachial LNs were harvested, single cell suspensions were prepared from collagenase-treated LNs, and stained with biotinylated anti-mouse CD11c mAb (HL3; PharMingen, San Diego, CA) and PerCP-streptavidin (PharMingen). Ratios of FITC$^+$ cells in CD11c$^+$ cells were analyzed to examine the migration of LC from skin to LNs using FACScan (Beckton Dickinson, Mountain View, CA). To assess the maturation of LCs, LN cells were stained with PE-anti-mouse CD40 mAb (3.23; Immunotech, Marseille Cerdex, France) or PE-anti-mouse CD86 mAb (RMMP-1; Immunotech), and expression of CD40 or CD86 on CD11c$^+$FITC$^+$ cells in LNs were analyzed.

T cell proliferation response

On day 5 after the sensitization with 3.0% TNCB, single cell suspensions were prepared from inguinal, axillary and brachial LNs, and T cells were purified by the depletion of B220$^+$ cells and Mac-1$^+$ cells using MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To prepare TNP-conjugated spleen cells, spleen cells from wild-type mice were depleted of Thy1.2$^+$, CD4$^+$ and CD8$^+$ cells on MACS columns. The cells were incubated with 100 mM trinitrobenzene sulfonate (TNBS; WAKO, Osaka, Japan) in PBS at 37°C for 5 min, and irradiated 35 Gy with ^{137}Cs. T cells from TNCB-sensitized mice (5×10^5 cells/well) were cultured with TNP-conjugated apleen cells (2×10^5 cells/well) in 200 µl RPMI1640 (SIGMA) containing 50 mM 2-mercaptoethanol (GIBCO BRL, Gaithersburg, MD), 50 µg/ml streptomycin (Meiji, Tokyo, Japan), 50 µg/ml penicillin (Meiji) and 10 % heated fetal calf serum (SIGMA) in a 96 well flat-bottom plate for 72 h at 37 °C in 5.0% CO2. After 72 h of culture, cultures were pulsed with [^{3}H]-thymidine (0.25 µCi/ml) (Amercham, Buckinghamshire, England) for 6 h. Cells were harvested with Micro 96 cell harvester (SKATRON, Lier, Norway) and incorporated [3H]-thymidine was measured with Micro Beta (Pharmacia Biotech, Piscataway, NJ). For OX40 expression, cells were cultured for 72 h and harvested. Cells were incubated with FITC anti-mouse CD4 mAb and PE anti-mouse OX40 (OX86) (Immunotech), and analyzed by FACS Calibur (Beckton).

CHS of mice transferred with T cells

T cells (2×10^7 cells/mouse) from TNCB-sensitized mice were suspended in PBS, and injected intravenously into a mouse. 12 h later, the outside of one ear was challenged with 25 µl of 1.0% TNCB, and another ear was applied with 25 µl vehicle. 24 h after the challenge with TNCB, ear swelling was measured as described above.

Induction of IP-10 mRNA expression by the injection with rIL-1 and rTNFα

100 ng of rIL-1α/β with or without anti-TNFα mAb (XT22, Endogen) and 100 ng of rTNFα was injected into the ear skin.   3 h after injection, total RNA was prepared from ear tissues, and IP-10 mRNA expression detected by Northern blot hybridization.

Northern blot hybridization analysis

24 h or 3 h after TNCB challenge or cytokine injection, total RNA was isolated from ear tissues and Northern blot hybridization was carried out as described elsewhere [44].

Statistics

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

Results

Importance of IL-1 and TNFα in CHS

To elucidate whether the activities of TNFα in IL-1α/β$^{-/-}$ mice and those of IL-1α/β in TNFα$^{-/-}$ mice compensate their deficient function mutually, CHS in IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice were examined.   Murine CHS is known to depend on the genetic background [19] and also on the sensitizing reagent [20-23].   Therefore, I first assayed ear swelling responses to 2, 4, 6-trinitrochlorobennzene (TNCB) and oxazolone in these mutant mice on C57BL/6J and BALB/cA background.   CHS against TNCB in IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice on C57BL/6J and BALB/cA background were similarly impaired significantly (Fig. 1).   The responses in IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice were impaired more severely than those in IL-1α/β$^{-/-}$ or TNFα$^{-/-}$ mice.   Similar results were obtained in CHS against oxazolone in these mutant mice (data not shown).   These results indicate that both IL-1 and TNFα play important roles in CHS, and also suggest that IL-1 and TNFα partially compensate each other for the role in CHS.

Effects of IL-1α/β- and TNFα-deficiency on the migration and maturation of LC

LC is known as major antigen presenting cells (APCs) for antigen-sensitization in CHS. IL-1α/β were shown to play an important role for the migration of LC from skin to draining LNs, but not for their maturation [9].   I labeled LC with FITC by painting the skin and examined the migration of FITC$^+$ LC to regional LNs in the gene-deficient mice by flow cytometry.   The migration of FITC$^+$ LC from skin to draining LNs was impaired in TNFα$^{-/-}$ mice as in IL-1α/β$^{-/-}$ mice both on C57BL/6J and BALB/cA backgrounds, and in IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice, the migration was impaired more severely (Fig. 2a and data for C57BL/6J background not shown).   Less than 5% of CD11c$^+$ cells in the LNs were labeled with FITC in LNs from IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice, whereas more than 40 and 20% of CD11c$^+$ cells were labeled with FITC in the LNs from wild-type C57BL/6J and BALB/cA mice, respectively (Fig. 2a and data for C57BL/6 background not shown).

There was no difference among IL-1α/β$^{-/-}$, TNFα$^{-/-}$, IL-1α/β$^{-/-}$xTNFα$^{-/-}$ and wild-type mice both on BALB/cA and C57BL/6J backgrounds in the expression of CD40 and CD86 molecules on FITC$^+$ CD11c$^+$ cells (Fig. 2b, and data for C57BL/6 background not shown). The densities of CD80 and ICAM-1 expression were also the cases (data not shown).

These findings indicate that TNFα also plays an important role in the migration of LC to LNs as does IL-1, and these two cytokines partly compensate each other in the function, and that neither IL-1 nor TNFα affects the maturation of LC at least in terms of
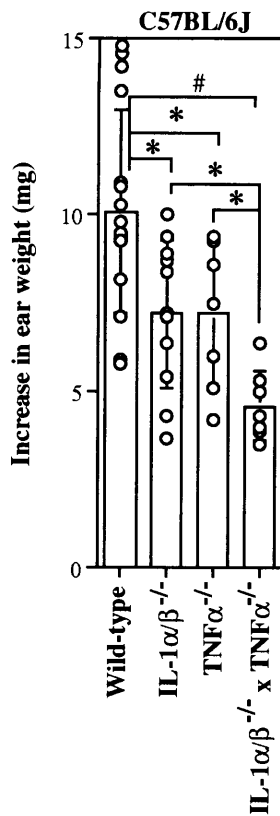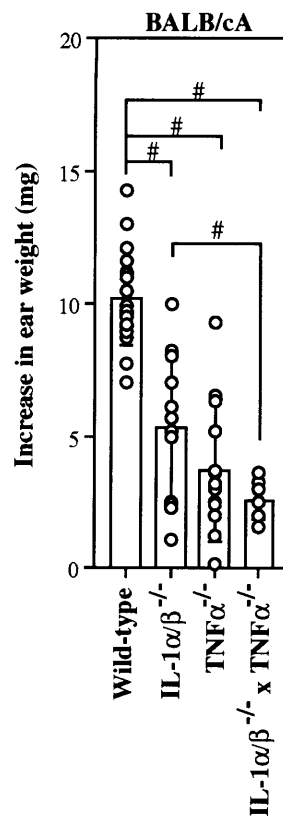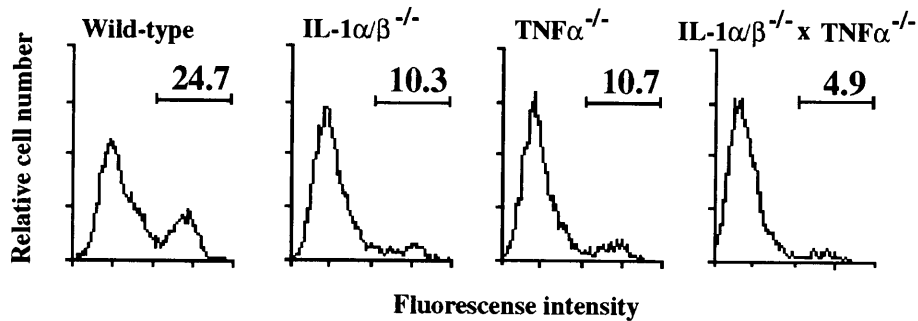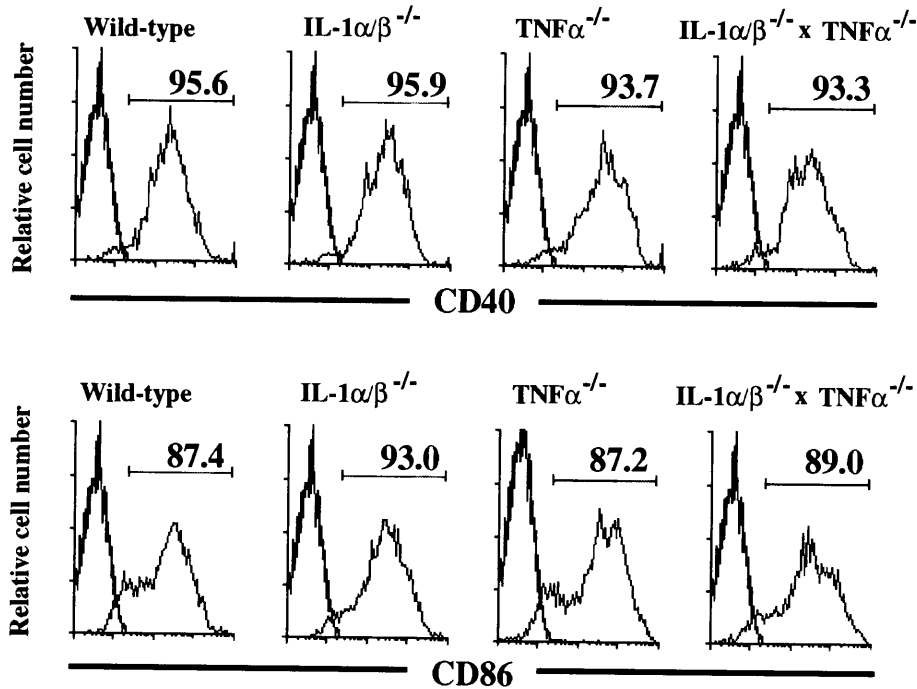
Figure. 1 Effect of IL-1- and TNFα-deficiency on TNCB-inducedCHS

Increment of ear swelling in TNCB-induced CHS in IL-1α/β-/-, TNFα-/- and IL-1α/β-/-xTNFα-/- mice. (A) TNCB-induced CHS using C57BL/6J wild-type (n=14), IL-1α/β-/- (n=11), TNFα-/- (n=7) and IL-1α/β-/-xTNFα-/- (n=7) mice.

(B) TNCB-induced CHS using BALB/cA wild-type (n=19), IL-1α/β-/- (n=14), TNFα-/- (n=13) and IL-1α/β-/-xTNFα-/- (n=7) mice. Each circle represents an individual mouse and average and standard deviation (SD) are shown. Student's t test was used for statistical evaluation of the results. * p < 0.05 and # p < 0.001

Figure. 2 Effect of IL-1- and TNFα-deficiency on LC migration and maturation
Mice were epicutaneously sensitized with 0.5% FITC. 24 h later, draining LNs were
harvested and analyzed for FITC (A) and CD40 and CD86 expression (B) by flow cytometry.
(A) Ratio of FITC+ cells among CD11c+ cells from wild-type, IL-1α/β-/-, TNFα-/- and
IL-1α/β-/-xTNFα-/- mice on BALB/cA background. (B) Expression of CD40 and CD86
among CD11c+ FITC+ cells from wild-type, IL-1α/β-/-, TNFα-/- and IL-1α/β-/-xTNFα-/-
mice on BALB/cA background. One representative result from four independent
experiments is shown.

the expression of cell surface molecules.

Effects of IL-1α/β- and TNFα-deficiency on hapten-specific T cell stimulation

Purified T cells from TNFα$^{-/-}$ and IL-1α/β$^{-/-}$ mice treated with TNCB were stimulated with TNP-conjugated splenocytes and assayed for their proliferative responses. The proliferation of T cells from TNCB-sensitized IL-1α/β$^{-/-}$ mice was lower than that of wild-type mice, which is consistent with my previous findings [9], whereas the response of T cells from TNCB-sensitized TNFα$^{-/-}$ mice was comparable to that of T cells from wild-type mice (Fig 3a). IL-1 is known to induce OX40 on CD4^{+} T cells [15], and OX40-OX40L co-signaling is required for hapten-specific T cell priming and the development of CHS [24]. Consistently with hapten-specific T cell proliferative responses, OX40 expression on T cells of IL-1α/β$^{-/-}$ mice was lower than that of wild-type mice, while that of TNFα$^{-/-}$ mice was comparable (Fig 3b). These results indicate that TNFα does not play a role in T cell priming with antigen.

To confirm the results mentioned above, I next carried out a cell transfer experiment. Non-sensitized wild-type mice were transferred with LN T cells from wild-type, IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice sensitized with TNCB, and challenged with TNCB, and then assayed for ear swelling. Ear swelling was impaired in the mice transferred with TNCB-sensitized IL-1α/β$^{-/-}$ T cells, but not with TNFα$^{-/-}$ T cells (Fig 3c). The impairment was observed in mice received IL-1α/β$^{-/-}$ T cells from either C57BL/6J or BALB/cA background mice (Fig 3b and data for BALB/cA background not shown).

These results indicate that TNFα is not required for the sensitization of T cells, although IL-1 is required.

Roles of IL-1 and TNFα in the elicitation phase of CHS

To elucidate whether IL-1 and TNFα involve in the mechanism of elicitation phase of CHS, I next carried out to transfer T cells from wild-type mice into the wild-type and mutant mice since IL-1-deficiency affect T cell-priming. T cells from wild-type C57BL/6J mice sensitized with TNCB were transferred into wild-type, IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice on C57BL/6 background, and these mice were challenged with TNCB. Ear swelling responses in IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice received wild-type T cells sensitized with TNCB were significantly impaired similarly compared with those in wild-type mice (Fig. 4a). The response in IL-1α/β$^{-/-}$ mice on BALB/c background received wild-type BALB/cA T cells were also significantly impaired (data not shown). These results indicate that both IL-1 and TNFα play important roles in the
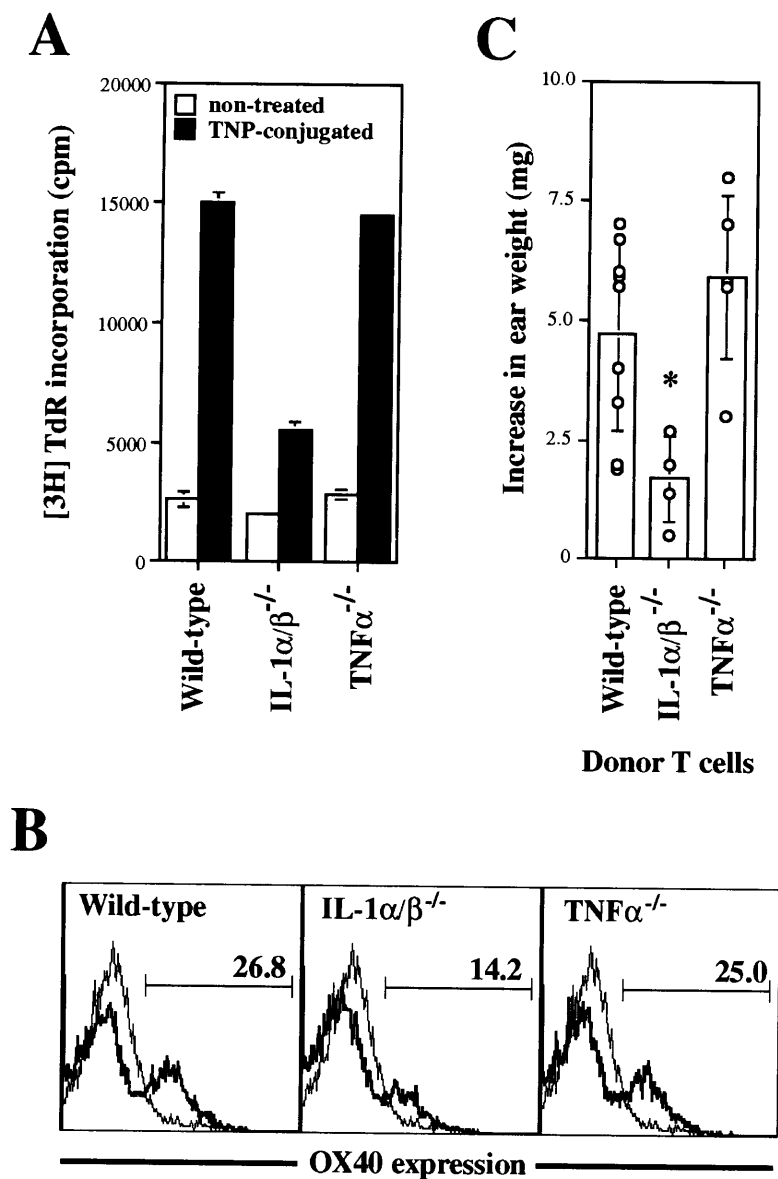
Figure. 3 Effect of IL-1- and TNFα-deficiency on hapten-specific T cell induction

On day 5 after sensitization with 3.0% TNCB, LNs were harvested and T cells were purified using MACS separation columns.

(A) LN T cells (5x10-5 cells) from TNCB-sensitized mice and irradiated TNP-conjugated APCs (2x10-5 cells) or non-treated APCs from C57BL/6 splenocytes depleted with T cells were cultured for 72 h. Proliferative response was assessed by the incorporation of [3H]-thymidine. One representative result from three independent experiments is shown.

(B) After 72 h of culture, cells were harvested and incubated with FITC anti-mouse CD4 mAb and PE anti-mouse OX40 mAb. The expression of OX40 on CD4+ T cells were assessed by FACS. Normal lines shows isotype matched control Ig staining and solid lines are the staining with anti-OX40 mAb. One representative result from three independent experiments is shown.

(C) LNs T cells (2x10-7 cells) from TNCB-sensitized wild-type, IL-1α/β-/- and TNFα-/- mice were injected intravenously into non-sensitized wild-type mice on C57BL/6 background.

5 days later, ear swelling in TNCB-induced CHS was measured as in Figure. 1. Wild-type mice injected with wild-type T cells (n=9), IL-1α/β-/- T cells (n=5) and TNFα-/- T cells (n=5) on C57BL/6 background. Each circle represents an individual mouse, and average and SD are shown. Student's t test was used for statistical evaluation of the results. * p < 0.05

elicitation phase of CHS and suggest that either TNFα by IL-1 or IL-1 by TNFα mediates the local inflammation since there was a similar degree of the reduced CHS among IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice.

In the next experiment, I examined which cytokines, TNFα or IL-1α/β, is in an upstream position in the pathway for the elicitation phase by the injection of rTNFα or rIL-1α/β into IL-1α/β$^{-/-}$ or TNFα$^{-/-}$ mice, respectively, after received TNCB-sensitized wild-type T cells. The IL-1α/β$^{-/-}$ mice received the T cells showed the strong response by the administration of rTNFα to the comparable level to that of wild-type mice injected with rTNFα, although the response of wild-type mice was also enhanced by the rTNFα injection (Fig. 4b). On the other hand, the response of TNFα$^{-/-}$ mice was not affected by the rIL-1α/β injection (Fig. 4c). These results suggest that TNFα is in a downstream pathway to IL-1 and plays an important role in the elicitation of CHS.

Effects of IL-1α/β- and TNFα-deficiency on the expression of various genes for adhesion molecules and chemokines in the tissue of CHS

I next investigated the expression of various genes for adhesion molecules and chemokines in ear tissues from IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice sensitized and challenged with TNCB. In adhesion molecules examined, the expression of ICAM-1 mRNA, but not mRNA for E-cadherin and E-selectin, was severely reduced in IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice (Fig. 5a and b). In chemokine mRNA, MCP-1 mRNA expression was not impaired in any of these deficient mice, Groα mRNA was reduced in expression only in IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice, and the expression of mRNAs for MIP-1α, MIP-1β and IP-10 was impaired in all mice deficient in IL-1α/β and/or TNFα (Fig. 5c and d). The expression of IP-10 mRNA was impaired more severe in TNFα$^{-/-}$ mice than in IL-1α/β$^{-/-}$ mice. I also examined IFN-γ mRNA expression because IFN-γ was reported to be an IP-10 inducer [25]. Interestingly, the expression was not detected in any mice examined although IP-10 mRNA expression in IL-1α/β$^{-/-}$ mice was detected (Fig. 5e and f). Similar results obtained in these deficient mice both on BALB/cA and C57BL/6J mice (Data for C57BL/6J mice not shown).

ICAM-1, MIP-1α and MIP-1β mRNA expression in the inflammatory site was reduced in both IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice, however, CHS was impaired only in IL-1α$^{-/-}$ mice (data on CHS not shown), consistent with my previous report [9]. On the other hand, IP-10 mRNA expression was reduced in IL-1α$^{-/-}$ and IL-1α/β$^{-/-}$ mice, but not in IL-1β$^{-/-}$ mice, in correlation with CHS (Fig. 6a and b). Furthermore, IP-10 mRNA expression in IL-
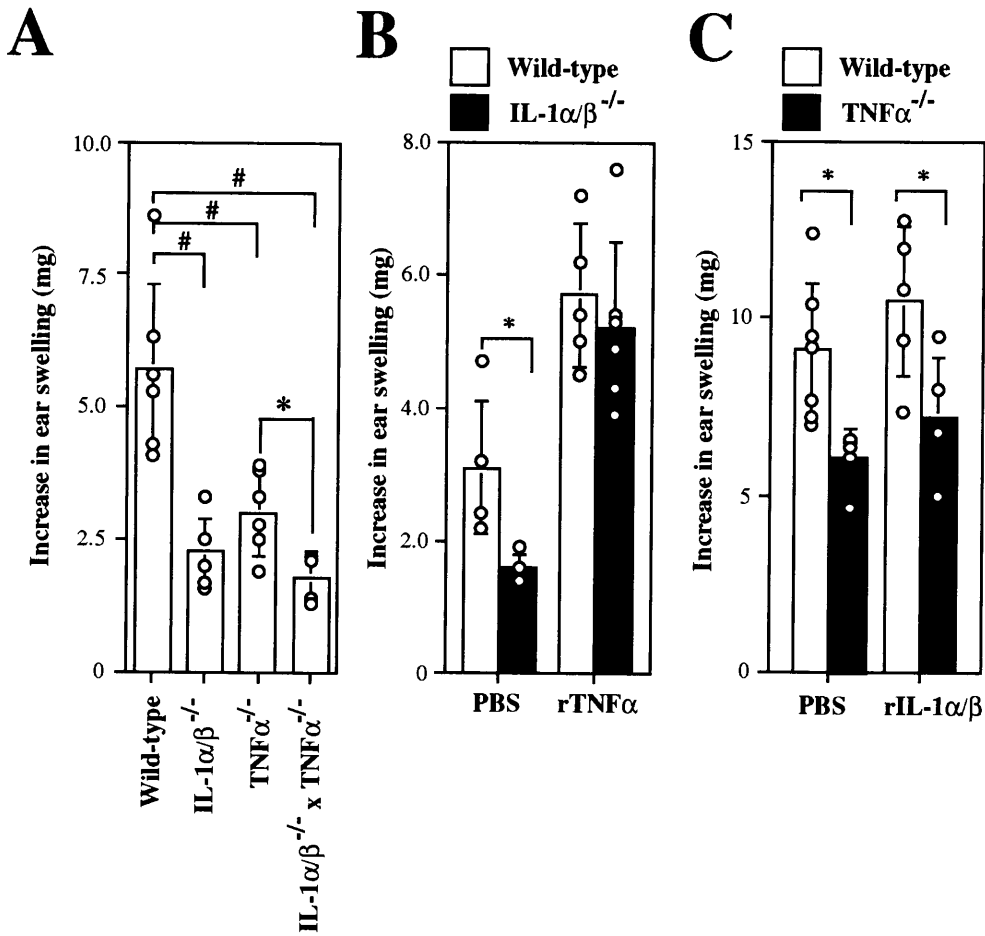
Figure. 4 Roles of IL-1 and TNFα in the elicitation phase of CHS

(A) TNCB-sensitized wild-type LN T cells were injected intravenously into non-sensitized wild-type (n=7), IL-1α/β-/- (n=5), TNFα-/-(n=6) and IL-1α/β-/-xTNFα-/- (n=4) mice on C57BL/6 background. On 5 days after injection, mice were challenged with 1.0% TNCB and ear swelling was measured after 24 h as in Figure 1. (B) TNCB-sensitized wild-type LN T cells were injected intravenously into non-sensitized wild-type and IL-1α/β-/- mice on C57BL/6 background. On 5 days after injection, mice were challenged with 1.0% TNCB and injected with 50 ng of rTNFα intradermally, and ear swelling was measured after 24 h as in Figure 1. Wild-type mice were treated with PBS (n=4) or rTNFα (n=5), and IL-1α/β-/- mice were treated with PBS (n=4) or rTNFα (n=6). (C) On 5 days after sensitization with 3.0% TNCB, wild-type and TNFα-/- mice on C57BL/6 background were challenged with 1.0% TNCB and injected 50 ng of rIL-1α/β intradermally, and ear swelling was measured after 24 h as in Figure 1. Wild-type mice were treated with PBS (n=4) or rIL-1α/β (n=4), IL-1α/β-/- mice were treated with PBS (n=5) or rIL-1α/β (n=5). Each circle represents an individual mouse, and average and SD are shown. Student's t test was used for statistical evaluation of the results. * p < 0.05, # p < 0.001.
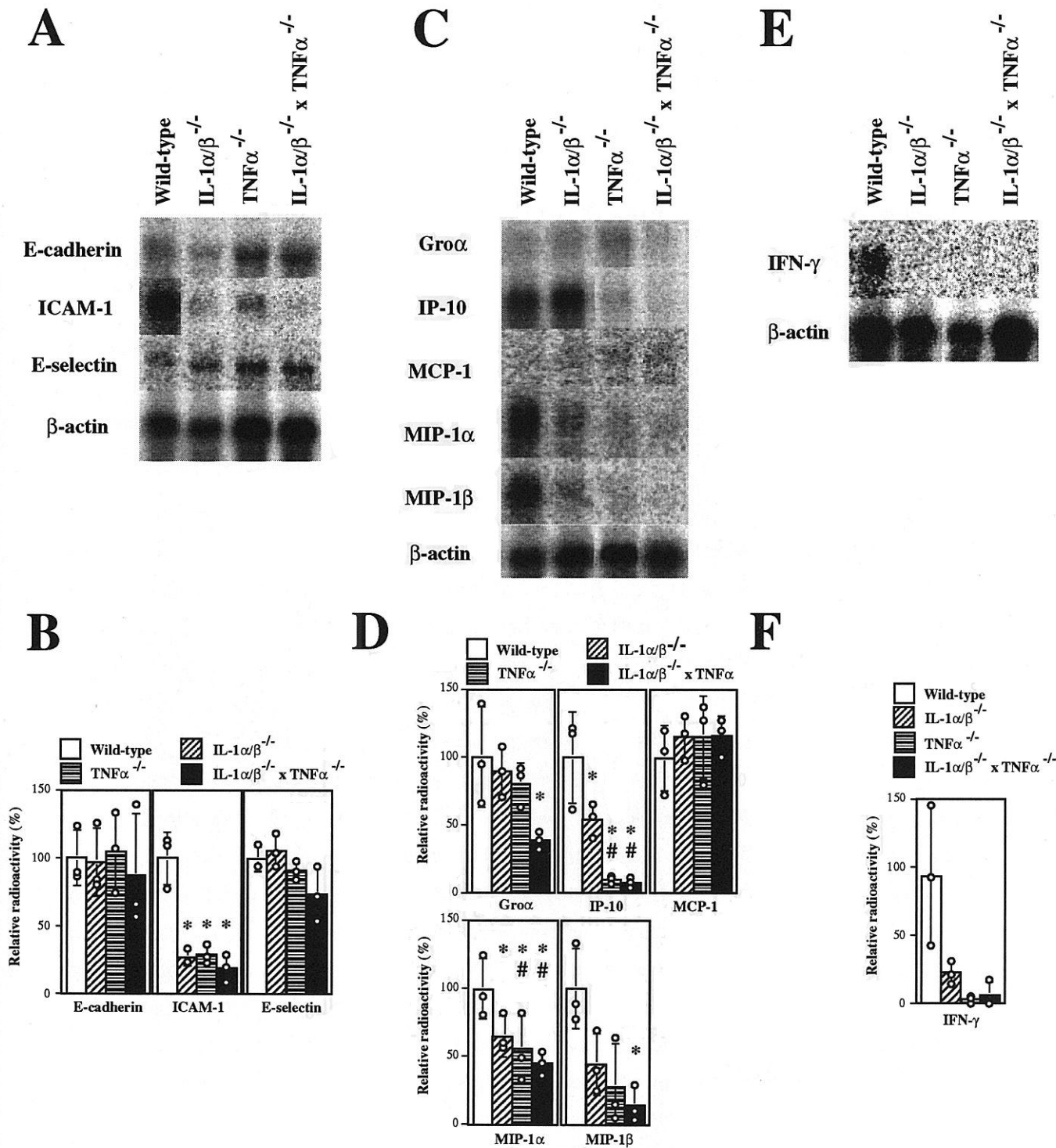
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Figure. 5 Effect of IL-1- and TNFα-deficiency on mRNA expression of adhesion molecules, chemokines and IFN-γ

Northern blot hybridization and relative mRNA levels to that of the wild-type control for the detection of adhesion molecules (A and B), chemokines (C and D) and IFN-γ (E and F) expression in TNCB-challenged ear skin of Wild-type, IL-1α/β-/-, TNFα-/- and IL-1α/β-/-xTNFα-/- mice on BALB/cA background as in Figure 1. (A,C and E) Northern blot analysis. (B, D and F) Relative radioactibites. * p < 0.05 vs wild-type mice and # p < 0.05 vs IL-1α/β-/- mice.
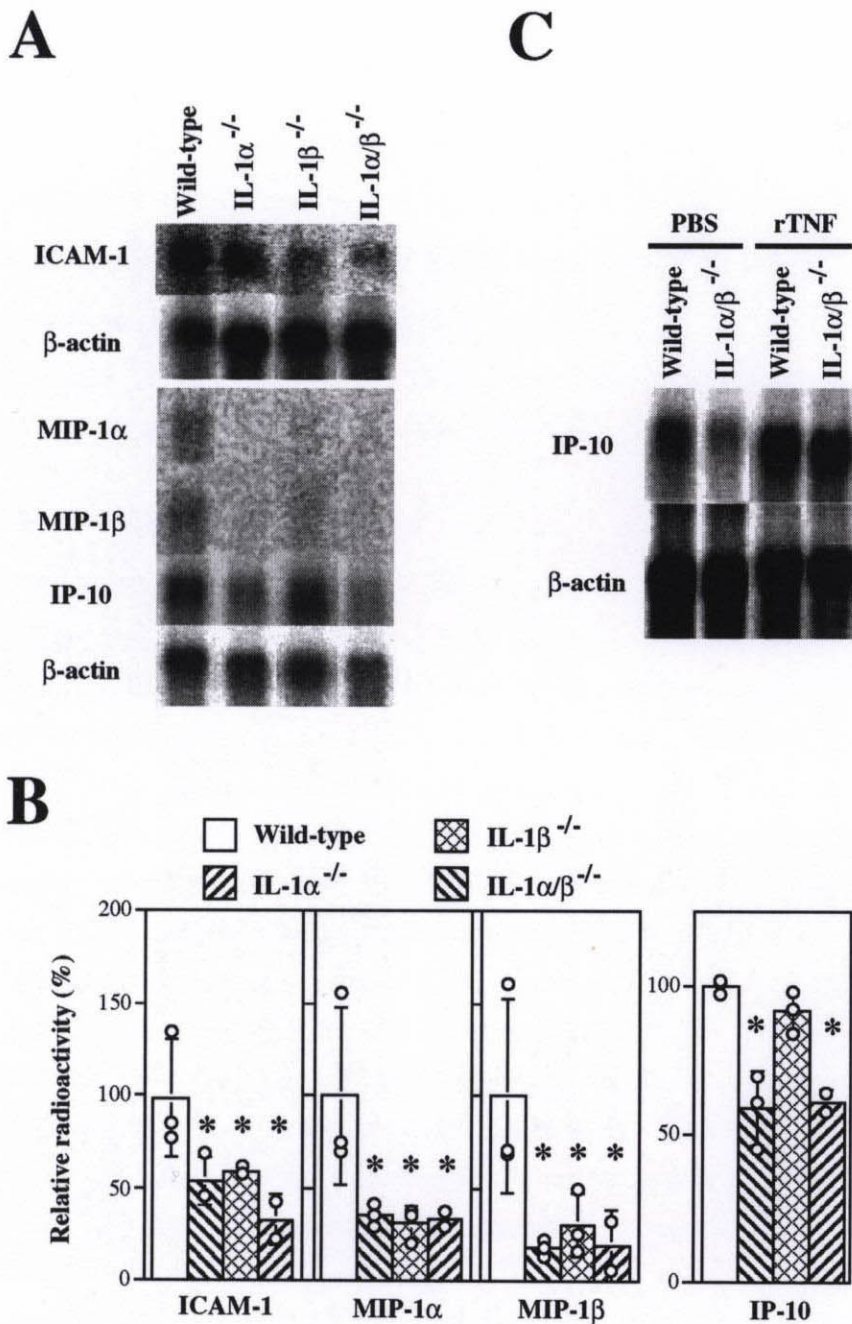
A

C

B

Figure. 6 mRNA expression of adhesion molecules and chemokines in IL-1α-/-, IL-1β-/- and IL-1α/β-/- mice

Northern blot hybridization and relative mRNA levels to that of the wild-type control for the detection of adhesion molecules and chemokines expression in TNCB-challenged ear skin of wild-type, IL-1α-/-, IL-1β-/- and IL-1α/β-/- mice on BALB/cA background as in Figure 1. (A) Northern blot analysis. (B) Relative radioactibies. * p < 0.05 vs wild-type mice. (C) The reduced IP-10 mRNA levels in the skin of IL-1α/β-/- mice challenged with TNCB was reconstituted by injection of rTNFα as shown in Figure 4C.

$1\alpha/\beta^{-/-}$ mice received wild-type T cells sensitized with TNCB and injected with PBS in Fig. 4b was lower than that in wild-type mice. As expectedly, that of rTNFα-treated IL-$1\alpha/\beta^{-/-}$ mice received wild-type T cells sensitized with TNCB was recovered to the level of that in rTNFα-treated wild-type mice received wild-type T cells sensitized with TNCB (Fig. 6c). Taken together, these results suggest that TNFα downstream of IL-1α upregulates elicitation of CHS by stimulating IP-10 expression.

Critical role of IP-10 in elicitation of CHS

To confirm the role of TNFα in IP-10 mRNA expression in the ear skin, I injected rTNFα intradermally into the ear of wild-type mice and IP-10 mRNA expression was examined. The rTNFα injection increased the IP-10 mRNA expression dose-dependently and this expression was mediated by both TNFRI and TNFRII (Fig. 7a and b). Moreover, IP-10 mRNA expression was induced, when rIL-1 or rTNFα was injected intradermally into the ears of IFN-$\gamma^{-/-}$ mice (Fig. 7c), and IP-10 mRNA expression induced by the rIL-1 injection was suppressed by the administration of anti-TNFα in the ear skin (Fig. 7c). I next injected rTNFα intradermally into ears of IL-$1\alpha/\beta^{-/-}$xTNF$\alpha^{-/-}$ mice, and found that IP-10 mRNA was strongly induced by the injection (Fig. 7d). To examine the role of IFN-γ in CHS, I examine CHS of IFN-$\gamma^{-/-}$ mice against TNCB. They showed a degree of CHS comparable to that in wild-type mice both on C57BL/6J and BALB/cA backgrounds (Fig. 7e) and IP-10 mRNA expression was also observed, but reduced partially, and TNFα mRNA expression was normal in their ear tissues (Fig. 7f). These results suggest that TNFα induces IP-10 mRNA expression in the ear skin through an IFN-γ-independent pathway.

To determine whether or not IP-10 is necessary for CHS, I examined the blockade of IP-10 signaling by anti-IP-10 mAb administration into the ear skin challenged with TNCB in the elicitation phase during CHS. CHS in mice treated with anti-IP-10 mAb was significantly reduced compared with that in mice treated with control Ig (Fig. 8a). This result indicates that IP-10 plays an important role in CHS. Moreover, to study whether the impaired CHS in TNF$\alpha^{-/-}$ mice was caused by the failure of IP-10 expression, the effect of rIP-10 on CHS was examined by an intradermal injection into the ear challenged with TNCB. The ear swelling of TNF$\alpha^{-/-}$ mice challenged with TNCB was restored by the injection to the level of wild-type mice, although ear swelling was not observed without TNCB challenge (Fig. 8b).

Taken all together, these results indicate that TNFα induced by IL-1 plays a critical role in the elicitation of CHS through induction of IP-10 expression independent of IFN-γ.
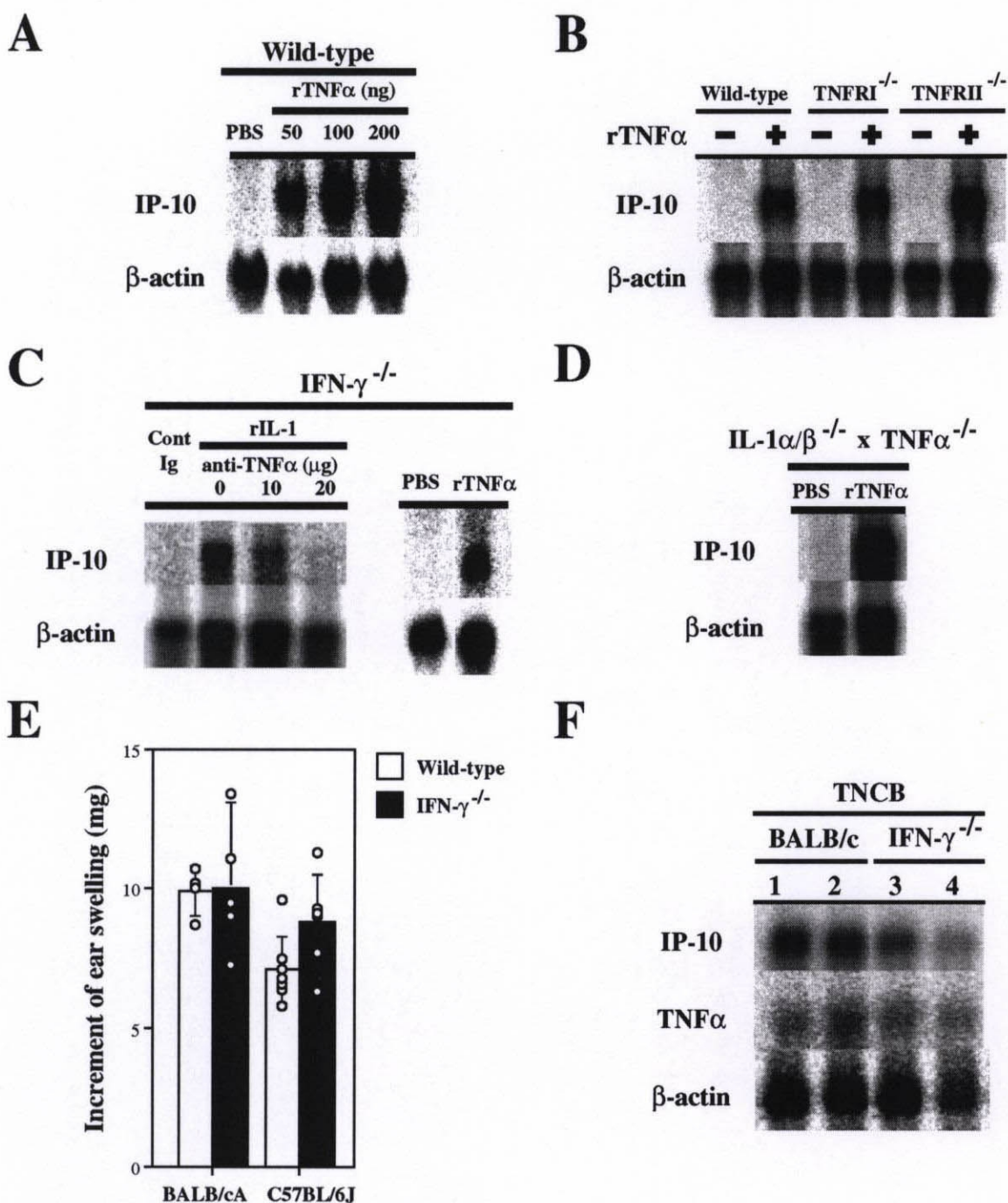
89

Figure. 7 IFN-γ-independent IP-10 production by TNFα

rIL-1 with or without anti-TNFα, rTNFa or PBS was injected intradermally into an ear of wild-type, IFN-γ-/- and IL-1α/β-/-xTNFα-/-, TNFRI-/- and TNFRII-/- mice. At 3 h after injection, total mRNA from the ear was prepared, and mRNA expression of IP-10 was assessed by northern blot analysis. (A) rTNFα was injected intradermally into an ear of BALB/cA wild-type mice. (B) 100 ng of rTNFα was injected intradermally into an ear of wild-type, TNFRI-/- and TNFRII-/- mice on C57BL/6 background. (C) 100 ng of rIL-1 with or without anti-TNFα and 100 ng of rTNFα was injected intradermally into an ear of IFN-γ-/- mice on BALB/cA background. (D) 100 ng of rTNFα was injected intradermally into an ear of IL-1α/β-/-xTNFα-/- mice on BALB/cA background. (E) TNCB-induced CHS using wild-type (n=6) and IFN-γ-/- (n=5) on BALB/cA background and wild-type (n=7) and IFN-γ-/- (n=6) mice on C57BL/6J background. Ear swelling was measured after 24 h as in Figure 1. Each circle represents an individual mouse and average and SD are shown. (F) Northern blot hybridization analysis of cytokine expression in TNCB-challenged ear skin of wild-type and IFN-γ-/- on BALB/cA background as in Figure 5.
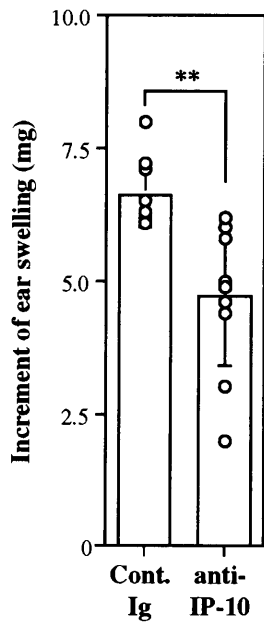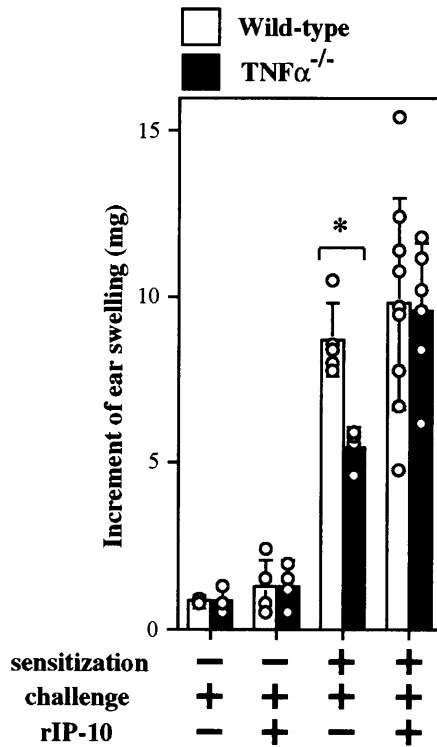
90

Figure. 8 The role of IP-10 in CHS

On 5 days after sensitization with 3.0% TNCB, mice were challenged with 1.0% TNCB and injected 100 μg of anti-IP-10 mAb or 1 mg of rIP-10 intradermally into the ear skin, ear swelling was measured as in Figure 1. (A) C57BL/6J wild-type mice was treated with control Ig (n=11) or anti-IP-10 mAb (n=11). (B) BALB/cA wild-type mice treated with PBS (n=6) or rIP-10 (n=8), TNFα-/- mice treated with PBS (n=5) or rTNFα (n=7). Each circle represents an individual mouse, and average and SD are shown. Student's t test was used for statistical evaluation of the results. * p < 0.05 and ** p < 0.001

91

Discussion

In this report, I showed clearly that IL-1α/β and TNFα have partially overlapping, but distinct roles in CHS. TNFα is not required for hapten-specific T cell priming in the sensitization phase of CHS, while IL-1α is necessary for this event[9]. In the elicitation phase, both IL-1 and TNFα involve. In this phase, IL-1 is necessary for TNFα induction, and TNFα plays an important role in IP-10 induction that is independent of IFN-γ-signaling.

It is known that the mechanism of CHS induction is different by using the distinct chemicals. In IL-4^{-/-} mice, the response to oxazolone was shown normally, however, that to TNCB, DNCB and DNFB was markedly impaired[23]. And, DNFB-induced CHS in IL-4^{-/-} mice on BALB/c background was reduced, but not on C57BL/6 background[19]. CHS in IL-1α/β[-/-] and TNFα^{-/-} mice was reduced against both TNCB and oxazolone (data not shown) and both on C57BL/6J and BALB/cA backgrounds. Therefore, the reduced CHS in these mice might not be caused by the kind of chemicals and mouse backgrounds

LC has an important role as a major antigen-presenting cells in hapten-specific T cell activation in the sensitization phase[1]. IL-1 and TNFα are known to involve in migration and maturation of LC from the skin to LNs. Recently, I showed that the degree of LC migration in IL-1α/β[-/-] mice was reduced temporally, however, the reduced LC migration was recovered gradually to that in wild-type mice[9]. Similar observation was obtained in TNFα^{-/-} mice (data not shown). Therefore, IL-1 and TNFα is necessary for LC migration, however, the reduced CHS in both IL-1α/β[-/-] and TNFα^{-/-} mice can not account for the reduced LC migration.

Recently, it has been reported that CD40-CD40L interaction is important for the induction of CHS by producing TNFα via CD40-CD40L crosslinking, resulting in promoting the migration and maturation of LC[26]. However, my results showed that functional LC maturation such as expression of activation surface marker and T cell-priming was normal in TNFα^{-/-} mice, and even the triple gene-deficiency of IL-1α, IL-1β and TNFα did not have an effect on maturation of LC. Therefore, it suggests that there is the other pathway exclude CD40-dependent TNFα signals to promote maturation of LC. GM-CSF produced by keratinocytes is one of the candidates in this question.

Consistently with my previous observation, IL-1 plays an important role in antigen-specific T cell activation[9,15]. On the other hand, it was shown that TNFα-deficiency did not affect antigen-specific T cell responses[27-29]. Moreover, although it was shown that TNFα was produced by CD4^{+} T cells by intracellular staining methods[30], I demonstrated that TNFα-deficiency of T cells did not have effect on the development of CHS by T cell-

adoptive transfer experiments.   These observations indicate strongly that IL-1 and TNFα have different roles in antigen-specific T cell activation in the sensitization phase of CHS.

In the elicitation phase, it is shown that ICAM-1 [16,31], MIP-1α and MIP1β [32] involves in ear swelling during CHS and expression of these genes was reduced in IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice.   However, ICAM-1, MIP-1α and MIP1β mRNA expression in the inflammatory site was reduced without suppression of ear swelling in IL-1β$^{-/-}$ mice.   In addition, CHS response was exacerbated in CCR5 (a receptor of MIP1β) deficient mice [33]. Therefore, the suppression of ear swelling in IL-1α/β$^{-/-}$ and TNFα$^{-/-}$ mice does not seem to be caused by the decreased mRNA expression of these genes.   In this screening by Northern blot analysis, the level of IP-10 mRNA was well correlated with the degree of ear swelling in IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$xTNFα$^{-/-}$ mice.

IP-10 plays an important role in the trafficking of effector T cells at sites of Th1 cell-mediated inflammation [34], and I showed here that IP-10 plays an important role in the elicitation phase of CHS using anti-IP-10 mAb.   It is known that IFN-γ is a potent inducer of IP-10 [25], and IP-10 mRNA is expressed by KC in the skin [2,35,36].   Recently, TNFα and IL-1β were also inducers of IP-10 in hepatocytes, Kupffer cells and endothelial cells [37].   I demonstrated here that the IP-10 induction was IFN-γ-independent- but IL-1-induced TNFα-dependent fashion in the skin.   In the support for this notion, other investigators showed that the ear swelling during CHS responses was suppressed in TNFα$^{-/-}$ and TNFRII$^{-/-}$ mice [4,18], but not in IFN-γRI$^{-/-}$ mice [38].   Moreover, the reduced CHS in TNFα$^{-/-}$ mice was recovered to the level of wild-type mice by rIP-10 injection.   CHS in IFN-γ$^{-/-}$ mice was normal, and that response in IFN-γ$^{-/-}$ mice may be explained for the normal expression of TNFα mRNA in the inflammatory site.   Thus, these observations indicate that TNFα is a main inducer of IP-10 in the skin during CHS induction.   On the contrary to my data, it was reported that IFN-γ-, but not TNFα-, induced IP-10 has a critical role in host defense for *Trypanosoma cruzi* infection [39].   On the other hand, it is known that TNFα can potentiate IP-10 mRNA expression in hepatocytes more strongly than IL-1β and IFN-γ while IP-10 mRNA levels in Kupffer cells and endothelial cells by these three cytokines were equivalent [37].   Therefore, there may be the different regulation of IP-10 induction by IL-1, TNFα and IFN-γ in the different producing cells or immune responses such as *Trypanosoma cruzi* infection and CHS responses.

IP-10, MIG and I-TAC are ligands for CXCR3.   Although I-TAC mRNA expression have not examined, the expression of MIG mRNA as well as IP-10 mRNA was abrogated in the TNCB-challenged site of TNFα$^{-/-}$ mice (data not shown).   However, IP-10 mRNA expression exhibited at 3 hrs after challenge with TNCB and reached the peak at

12 hrs, while the time kinetics of MIG mRNA expression was more delay than that of IP-10 mRNA expression (data not shown). Similar results observed in *Toxoplasma gondii* infection and IP-10 rather than MIG plays important roles in this infection [34]. In the skin, it was reported that IP-10 expression was shown on epidermal cells while MIG was expressed on dermal cells [40]. IP-10 rather than MIG may also play important roles in CHS since inflammatory cells infiltrate mainly into epidermis during CHS.

Although CHS is known as the T cell-mediated immune responses, various inflammatory cells such as neutrophils and macrophages are infiltrated in the inflammed region [1]. Recently, it was reported that mast cell-derived TNFα and macrophage inflammatory protein-2 (MIP-2) played an important role in the infiltration of polymorphonuclear leukocytes (PMNs) in CHS [41]. CHS were induced normally in mast cell-deficient Kit^w/Kit^{w-v} mice recieved wild-type mast cells compared with wild-type mice. However, these responses were not restored in mast cell-deficient Kit^w/Kit^{w-v} mice recieved TNFα$^{-/-}$ mast cells [41]. It was known that IL-1 can potentiate Th2 cytokine and MIP-2 production in activated mast cells [41,42], suggesting that not only KC but also mast cells are one of the producers of TNFα by IL-1 in CHS and that the suppressed CHS in mast cell-deficient Kit^w/Kit^{w-v} mice recieved TNFα$^{-/-}$ mast cells may be caused by the defect of TNFα-dependent IP-10 induction to infiltrate activated T cells in the challenged region. My results and these observations are suggested that IL-1-induced TNFα has a critical role in the infiltration of activated T cells and PMNs through IP-10 and MIP-2 induction in the elicitation phase of CHS.

I showed clearly here that IL-1 and TNFα has a distinct role in the sensitization and elicitation phases of CHS. My findings provide an important insight into molecular mechanisms of CHS development through the network of cytokines and chemokines.

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CHAPTER V

Impaired Allergen-Specific T Cell Responses and

B Cell Antibody Production

in Interluikin-17-Deficient Mice

Summary

Interleukin-17 (IL-17) is a proinflammatory cytokine produced by T cells. Since IL-17 is detected in sera from asthmatic patients and synovial fluids from arthritis patients, involvement in various human diseases has been suspected. In this study, I have generated IL-17-deficient (IL-17$^{-/-}$) mice, and investigated roles of IL-17 in various disease models. In these mice, contact and delayed-type hypersensitivity responses were significantly reduced, and airway hypersensitivity response was also reduced. These impaired responses might be caused by that allergen-specific T cell priming and T-dependent antibody production were severely impaired in these mutant mice. On the other hand, IL-17-deficiency of donor T cells did not affected on the development of acute graft-versus-host reaction. These findings indicate that IL-17 plays important roles in allergen-specific T cell-mediated immune responses.

Introduction

Interleukin-17 (IL-17) is a T cell-derived proinflammatory cytokine originally identified as cytotoxic T lymphocyte associated antigen-8 (CTLA-8) [1]. Murine IL-17 is a 21 kDa glycoprotein consisting of 147 amino acids, which has 63% amino acid homology with human IL-17 (155 amino acids) [2]. It has no obvious homology with other cytokines, but has 57% homology with the predicted amino acid sequence of the open reading frame 13 (ORF13) of *Herpesvirus saimiri* (HVS) (also called vIL-17) [1]. Recently, human IL-17 is found to form a novel cytokine family consisting of IL-17B, IL-17C, IL-17E and IL-17F other than IL-17 and vIL-17, and their identity with IL-17 is 16-50% [3-8]. A receptor for IL-17 (IL-17R) has been identified with no homology to any other cytokine receptor families and its mRNA expression shows ubiquitous tissue distribution [9]. 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 [10].

IL-17 is produced by $TCR\alpha/\beta^+CD4^-CD8^-$ thymocytes, activated $CD4^+$ and $CD4^+CD45RO^+$ memory T cells [2,11]. Although activated $CD8^+$ and $CD8^+CD45RO^+$ memory T cells are also known to produce IL-17 in human [12,13], its expression is restricted on $CD4^+$ T cells in mouse [14]. It is reported that IL-17 is produced by Th0/Th1, but not by Th2 cell clones established from rheumatoid arthritis patients, while it is produced by both Th1 and Th2 cell clones from human skin-derived nickel-specific T cells [15,16]. On the other hand, IL-17 is produced by T cells expressing $TNF\alpha$, but not by Th1 or Th2 cells, in mouse [14].

IL-17 has pleiotropic activities including induction of $TNF\alpha$, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-6, IL-8, IL-10, IL-12, GM-CSF, RANTES, MCP-1, MIP-2, Groα, LIF, stromelysin, PGE2, C3 and factor B on various cells such as human macrophages, fibroblasts and some epithelial cells [17-22], up-regulation of ICAM-1 and HLA-DR expression on kerationocytes [23], induction of iNOS and cyclooxygenase-2 (cox-2) on chondrocytes [24], cox-2-dependent PGE2-mediated osteoclast differentiation factor (ODF) expression on osteoblasts [25] and SCF and G-CSF-mediated granulopoiesis [26]. IL-17 acts on T cells as a co-stimulatory factor [9], enhances allorejection via promotion of dendritic cell (DC) maturation [27] and promotes tumor rejection by

activation of NK cells [28].   It is also known that IL-17 is detected in sera or the diseased parts of various patients, suggesting its involvement in the development of various human diseases such as osteoarthritis [29], rheumatoid arthritis [15,25,30], Lyme arthritis [14], multiple sclerosis [31], systemic lupus erythematosus [32], allograft rejection [27], lung inflammation and asthma [33,34], *Helicobacter pyloli* infection [35], acute leukemia [36], ischemia [37,38] and tumor rejection with angiogenesis [39,40].   However, pathological and physiological roles of IL-17 in a body have not been well understood.   In this report, I have generated IL-17-deficient (IL-17$^{-/-}$) mice to elucidate roles of IL-17 in various inflammatory diseases and immune responses.   Using IL-17$^{-/-}$ mice, I show that IL-17 is involved in diseases such as contact hypersensitivity (CHS), delayed-type hypersensitivity (DTH) and airway hypersensitivity response (AHR), but not in acute graft-versus-host reaction (GVHR).

Materials and Methods

Generation of IL-17^{-/-} mice

Genomic DNA including *il-17* gene was isolated from mouse 129/SVJ genomic phage library. A targeting vector was constructed to replace 2.1 kb genomic fragment with 2.5 kb DNA fragment containing EGFP gene and neomycin resistance gene (neo) under the control of phosphoglycerate kinase (PGK) 1 promoter between loxP and loxP sequences. The replaced genomic fragment contained from initiation codon ATG of first exon to second exon corresponding to 45-290 nucleotides of IL-17 cDNA. For negative selection, a diphtheria toxin A (DT) gene under the MC1 promoter was ligated at 5' end of targeting vector. The linearized targeting vector was electroporated into ES (E14.1) cells and selected in G418. Homologous recombination was screened by Southern blot analysis using 5' and 3' probes. Two clones of seven independently identified targeted ES clones were treated with *cre*-carried Adeno virus vector to delete neo gene. Chimera mice were generated by the aggregation method using C57BL/6J blastocysts as described elsewhere [57]. Chimera mice were mated with C57BL/6J female mice for germline transmission. For experiments, heterozygous mice were intercrosses to obtain homozygous mice (129xB6 F1) or backcrossed to C57BL/6J mice for four generations. The genotyping of IL-17^{-/-} mice for PCR primers was as described below: Primer 1; 5'-ACTCTTCATCCACCTCACACGA-3', Primer 2; 5'-GCCATGATATAGACGTTGTGGC-3'. Primer 3; 5'-CAGCATCAGAGACTAGAAGG GA-3'.

Cell preparations and cultures

Cell preparation and mitogenic response were performed as described previously [58,59].

ELISA for cytokines and antigen-specific Igs

For the detection of IL-17, monoclonal rat anti-mouse IL-17 and polyclonal biotinylated goat anti-mouse IL-17 as capture and detection Ab, respectively, were obtained from DAKO. For the detection of IL-1α, IL-1β and TNFα, hamster anti-mouse IL-1α mAb (Genzyme), hamster anti-mouse IL-1β mAb (Genzyme) and anti-

mouse TNFα mAb (ENDOGEN) as capture Abs and polyclonal rabbit anti-mouse IL-1α (Genzyme), polyclonal rabbit anti-mouse IL-1β (Genzyme) and rabbit anti-mouse TNFα (provided by Dr. Katsuo Noguchi, Teikyo University, Japan) as second antibodies were used. As third antibody, HRP-conjugated goat anti-rabbit IgG was purchased from Zymed. HRP-avidin was obtained PharMingen and TMB substrate was purchased from DAKO. Mini Kit™ mouse IL-4 Kit (ENDOGEN) for IL-4, Titer Zyme EIA kit (PerSeptive Diagnostics, Inc., Cambridge, MA) for IL-5 and OptEIA™ mouse IFN-γ set (BD PharMingen) for IFN-γ were used, and there levels were determined by following the manufacture's protocol.

TNP-, mBSA-, and OVA-specific Ig levels were measured by ELISA as described elsewhere [48,58,60].

CHS response

TNCB-induced CHS, LC maturation assay, TNP-specific T cell responses were assayed as described previously [48]. Ear swelling is calculated as follows; [Increment of ear swelling (mg)] = ([weight of challenged ear (mg)] - [weight of vehicle-treated ear (mg)]). Data shown are from time point of a maximal response. For DNFB-induced CHS, DNP-specific T cell responses and adoptive transfer analysis, it was performed as described elsewhere [61].

Vascular permeability assay

Vascular permeability was performed and modified as described previously [62]. At 5 min after challenge with 1.0% TNCB, 8.3 µl/g body wight of 1.0% Evan's 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 and minced in 150 µl of 0.5% Na2SO4. Evans' blue dye in the minsed tissues was extracted in 350 µl of acetone overnight and the absorbance at 650 nm was measured.

MPO assay

A 6 mm-diameter biopsy was taken from TNCB- or vehicle-challenged ear and homogenized, and MPO activity was measured as described elsewhere [63].

DTH response

mBSA-induced DTH was examined as described previously [64]. The degree of footpad swelling was caliculated as described below: [footpad swelling (%)] = {[footpad thickness of mBSA-injected footpad (mm)] − [footpad thickness of PBS-injected footpad (mm)]} ÷ [footpad thickness of PBS-injected footpad (mm)] × 100. Data shown are from time point of maximal response. For mBSA-specific LN cell responses, LN cells (4×10^5 cells/well) were cultured in the absence or presence of 40 μg/ml of mBSA for 3 days, followed by incorporation of [^{3}H]-thymidine (0.25 μCi/ml) (Amercham, Buckinghamshire, England) for 6 h. Then, cells were harvested with a Micro 96 cell harvester (SKATRON, Lier, Norway) and radioactivity was measured with Micro Beta (Pharmacia Biotech, Piscataway, NJ). Data shown are from time point of a maximal response.

AHR

For OVA/Alum-induced AHR, mice were sensitized with 100 μg/ml OVA/Alum intraperitoneally on day 0 and 12. 21 days later after a last sensitization, mice were challenged with 100 ng of OVA/PBS intranasally for 3 days. For OVA/PBS-induced AHR, IL-17$^{+/-}$ or IL-17$^{-/-}$ crossed on DO11.10 Tg mice were inhaled 100 ng of OVA/PBS intranasally for 4 days. 24 h after a last inhalation, AHR response to methacholine was measured with the Buxco as system described elsewhere [50].

Acute GVHR

Single cell suspension from spleen of IL-17$^{+/+}$ or IL-17$^{-/-}$ mice on C57BL/6J background. 6×10^7 spleen cells were injected into C57BL/6×BALB/c F1 (CBF1) mice intravenously. In an indicated time point after injection, spleen from recipient CBF1 mice was harvested and single cell suspension was prepared. Cells were incubated with anti-mouse CD16/CD32 (2.4G2), and then, stained with PE anti-mouse CD4 (RM4-5), PE anti-mouse CD8 (53-6.72) or PE anti-mouse CD45R/B220 (RA3-6B2) with FITC anti-mouse H-2K^d (SF1-1.1). All antibody was purchased by BD PharMingen. Cell population was analyzed by FACS Calibur. Alive cells were selected by staining with 7-actinomycin D (SIGMA). 10 days after transfer, spleen cells (2×10^6 cells/well) were cultured in the absence or presence with 2μg/ml ConA, and

proliferation levels were examined as described above. For CTL assay, to purify H-2K^{d}CD8$^+$ T cells used as effector cells, spleen cells of CBF1 received IL-17$^{+/+}$ or IL-17$^{-/-}$ spleen cells were passed through nylon wool columns. And then, cells were incubated with biotinylated anti-mouse H-2K^d (SF1-1.1), anti-CD4 beads, anti-B220 beads, anti-DX5 beads, anti-Mac-1 beads and anti-Ter119 beads and it was passed through MACS columns after the incubation with streptavidin-beads. P815 cells as target cells was used and labeled with ^{51}Cr by standard protocols.

Histology

Ears in TNCB-induced CHS (24 h after the challenge), footpads in mBSA-induced DTH (24 h after the challenge) and lungs in OVA-induced AHR (24 h after the last inhalation) were fixed in 10% formalin/PBS. Footpad samples were decalcified by Plank-Rychlo method and each sample was embedded in praffin. Praffin sections were stained with hematoxylin-eosin.

Statistics

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

Results

Generation of IL-17-deficient mice

I generated mice lacking the IL-17 expression by replacing exon 1 and 2 of *il-17* 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 the detection by ELISA (Figure 2C). IL-17$^{-/-}$ mice were generated from the crosses 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 co-stimulatory factor [9]. 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 (Figure 2A and B), although IL-17 production was completely abolished (Figure 2C). Proliferative responses of whole spleen cells to other mitogenic stimuli such as Con A, PHA and PMA plus ionomycin were also shown normal in IL-17$^{-/-}$ mice (Figure 2D). Therefore, these results indicate that IL-17 is not necessary for the cell proliferation and IL-4 and IFN-γ production by T cells in response to non-specific mitogenic stimuli.

CHS response

It was shown that nickel-specific skin derived T cells produce IL-17, and this IL-17 induces IL-6 and IL-8 production and ICAM-1 and HLA-DR expression on keratinocytes in human [23]. To elucidate the role of IL-17 in hapten-specific skin diseases, I investigated CHS responses in IL-17$^{-/-}$ mice. DNFB- and TNCB-induced
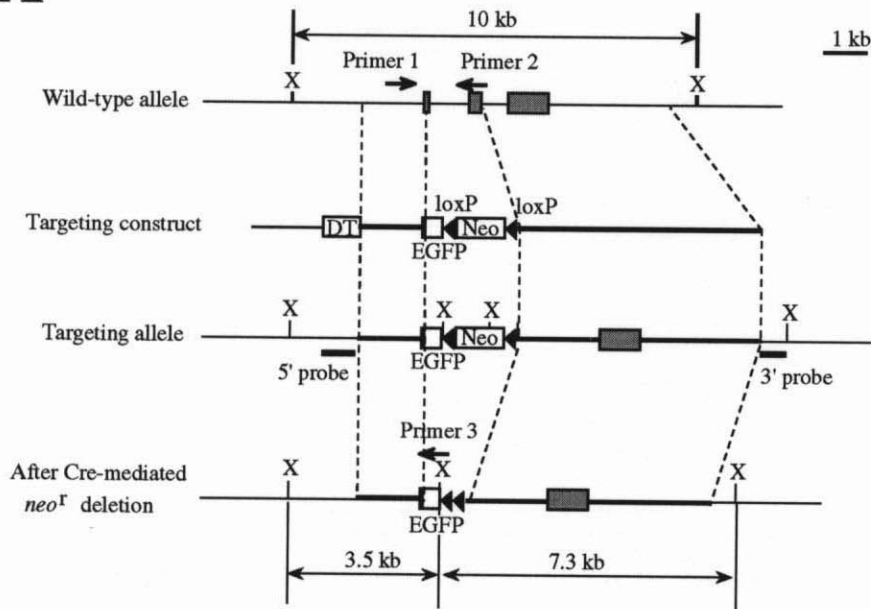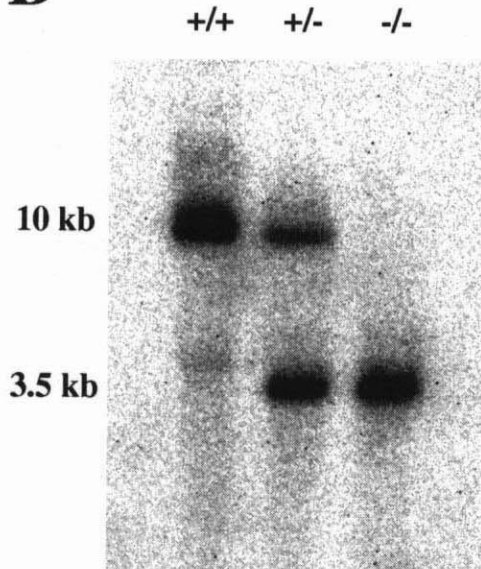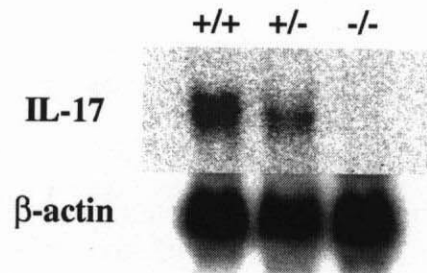
Figure 1. Generation of IL-17-/- mice
(A) Structure of the mouse il-17 locus (Wild-type allele), the IL-17 targeting construct (Targeting construct) and the predicted mutated il-17 gene before (Targeting allele) and after neomycin resistance gene (neor) deletion (After Cre-mediated neor deletion). Exons are represented by boxes. Exon 1 and 2 of il-17 gene were replaced with EGFP gene contained neor between loxP and loxP sequences. A diphtheria toxin A (DT) gene is external to the 5' genomic fragment for negative selection. The genomic probe for Southern blot analysis was used the fragment that lies external homologous regions used in the targeting construct. For ES cell screening, Southern blot analysis used the introduction of EGFP-neomiycin cassette Xba I(X) site. Primer 1, 2 and 3 were used in genotyping of mice. (B) Southern blot analysis of thymus DNA from IL-17 wild-type (+/+), heterozygous (+/-) and mutant (-/-) littermates. Using 5' probe, the endogenous (10 kb) and targeted (3.5 kb) bands were shown. (C) Northern blot analysis of IL-17 mRNA from splenic T cells stimulated with plate-coated anti-CD3 plus anti-CD28 mAb for 48h.
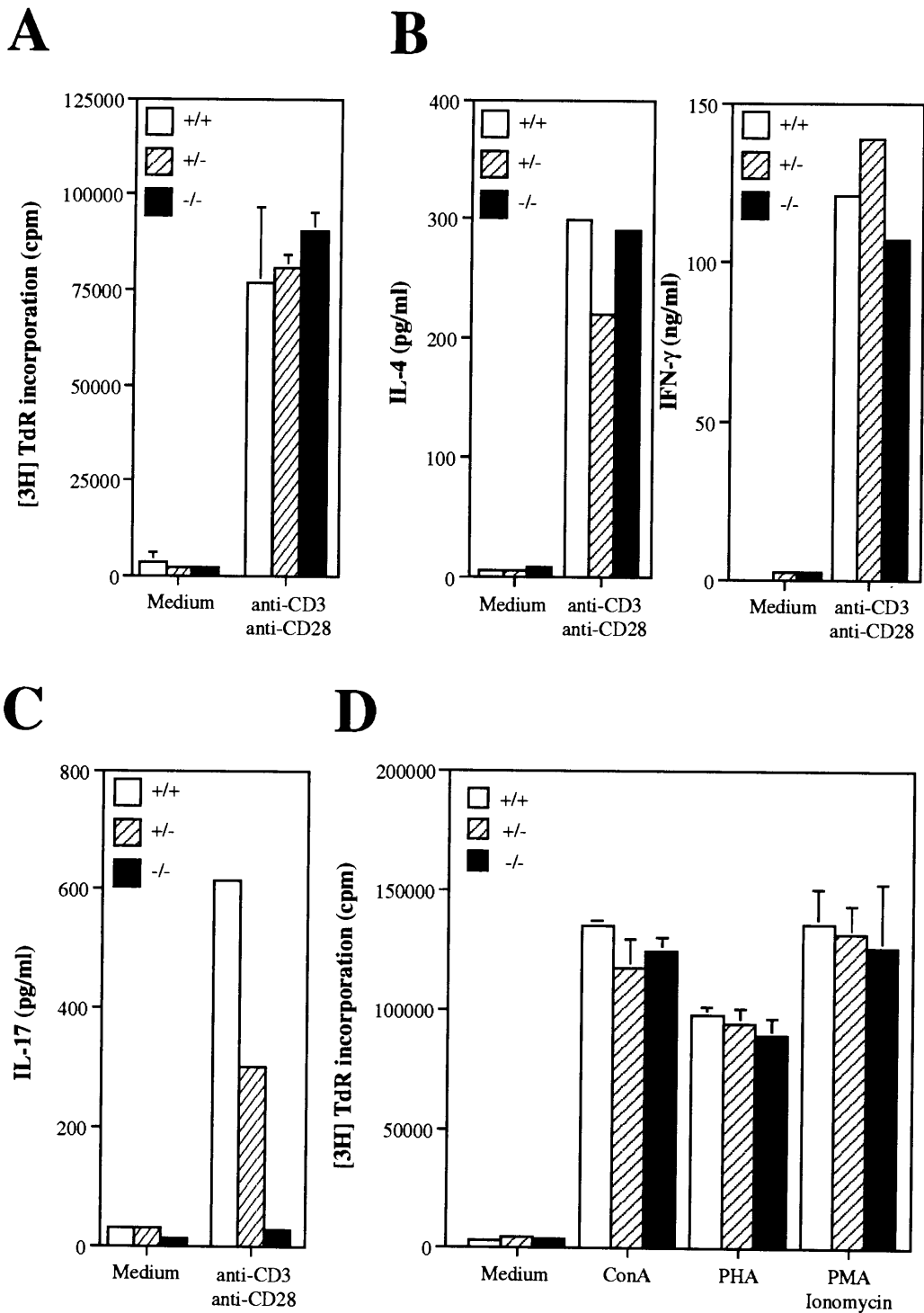
Figure 2. T cell functions to mitogenic stimuli in IL-17-/- mice

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 culture supernatants to plate-coated anti-CD3 plus anti-CD28 mAb stimulation for 48 h and proliferative responses to indicated stimuli (D) for 48 h were shown. Average ± SD of triplicate experiments is shown in the proliferative response, and cytokine levels in pooled triplicate supernatant from proliferative response assay were determined by ELISA. These results were reproducible two independent experiments.

CHS responses in IL-17$^{-/-}$ mice were markedly suppressed compared with those in IL-17$^{+/+}$ mice (Figure 3A and B). 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 [27]. Langerhans cells (LC), which are the major antigen-presenting cells in CHS induction, are immature in the skin and maturate during the migration process from the skin to draining LNs after activation with antigens [41]. However, LC migrating into draining LNs from the skin expressed a cell surface activation marker, CD40, normally in IL-17$^{-/-}$ mice after painting with FITC on the skin (Figure 3D). 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 (Figure 3E and F). Among the CD3^{+} cells, CD4^{+} T cells, but not CD8^{+} T cells, showed reduced proliferative response against DNBS (Figure 3G). IFN-γ production after stimulation with DNBS was markedly reduced in IL-17$^{-/-}$ T cell culture, while IL-4 production was hardly detected both in the culture of IL-17$^{+/+}$ and IL-17$^{-/-}$ T cells (Figure 3F). When TNCB-sensitized T cells were transferred into non-treated mice, CHS response in mice received T cells from IL-17$^{-/-}$ mice was significant low compared with that in mice received T cells from IL-17$^{+/+}$ mice (Figure 3H). Therefore, these results indicate that IL-17 is required for hapten-specific T cell priming, especially for CD4^{+} T cells, but not for LC maturation in the sensitization phase of CHS response.

In the elicitation phase of CHS, various cells such as neutrophils, macrophages and T cells were infiltrated in the challenged region with chemicals. In this phase, vascular permeability is important for the cell infiltration. Thus, I investigated vascular permeability in IL-17$^{-/-}$ mice, since IL-17 is suggested to be involved in this process through the induction of iNOS and cox-2. One hour after the challenge with TNCB, exudation of Evans blue dye in the ear was normal in IL-17$^{-/-}$ mice (Figure 3I).

To assess the effect of IL-17-deficiency on the expression of proinflammatory cytokines, chemokines and ICAM-1, protein or mRNA levels were analyzed by ELISA or Northern blot hybridization analysis, respectively. Proinflammatory cytokine, IL-

1α, IL-1β and TNFα productions in the lysate of ear challenged with TNCB in IL-17$^{-/-}$ mice was similar kinetics and levels compared with those in IL-17$^{+/+}$ mice (Figure 3J and K), although IL-17 was not produced in IL-17$^{-/-}$ mice (Figure 3J). On the other hand, mRNA levels of chemokines were reduced 20-50% in IL-17$^{-/-}$ mice compared with IL-17$^{+/+}$ mice at 6 h after TNCB challenge (Figure 3L). ICAM-1 expression was also decreased partially in IL-17$^{-/-}$ mice (Figure 3L). Then, infiltration levels of neutrophils were estimated by MPO assay (Figure 3M). Results show the involvement of IL-17 in this process, as suggested by a previous report [18]. Consistently with this observation, expression of chemokine mRNAs of Groα, MIP-1 and MIP-2 were reduced (Figure 3L). The pathological activities of TNFα and IL-1α/β are similar to that of IL-17, and in recent, it was reported that IL-17 is expressed by T cells producing TNFα [14]. These results show that IL-17 also plays an important role in the infiltration process of inflammatory cells in the elicitation phase of CHS response. To determine whether the roles of IL-17 in CHS response are different from those of TNFα because TNF production in the inflammatory site was normal in IL-17$^{-/-}$ mice and IL-17 production on T cells of TNFα$^{-/-}$ mice was also normal (data not shown), I examined CHS responses using IL-17$^{-/-}$xTNFα$^{-/-}$ mice. CHS responses in TNFα$^{-/-}$ mice as well as IL-17$^{-/-}$ mice were similarly reduced compared with those in IL-17$^{+/+}$xTNFα$^{+/+}$ mice, and those in IL-17$^{-/-}$xTNFα$^{-/-}$ mice was severely reduced compared with those in TNFα$^{-/-}$ or in IL-17$^{-/-}$ mice, respectively (Figure 3N). These results suggest that the reduced CHS response in IL-17$^{-/-}$ mice is caused by the TNFα–independent mechanism.

DTH response

I examined a role of IL-17 in DTH response, which is known as a Th1 cell-mediated cellular immune response. Methylated-BSA (mBSA)-induced DTH response in IL-17$^{-/-}$ mice was suppressed to ~80% of the average response of IL-17$^{+/+}$ mice (Figure 4A). In these mice, cell infiltration to the inflammatory site was milder than that in IL-17$^{+/+}$ mice (Figure 4B). T cell proliferative response of IL-17$^{-/-}$ mice against mBSA was reduced compared with those of IL-17$^{+/+}$ mice (Figure 4C). Consistently with the proliferation levels, IFN-γ production in culture supernatants from IL-17$^{-/-}$ mice was also lower than that from IL-17$^{+/+}$ mice (Figure 4C). IL-4 levels were too low to in both IL-17$^{+/+}$ and IL-17$^{-/-}$ culture supernatants (Figure 4C). These results
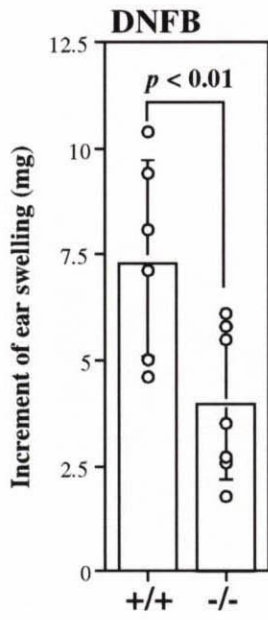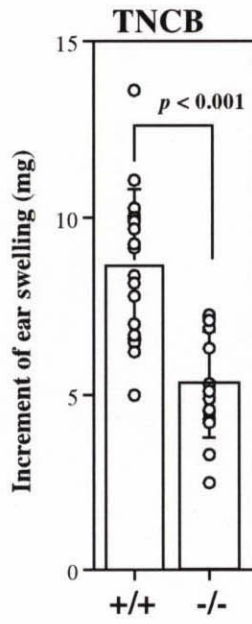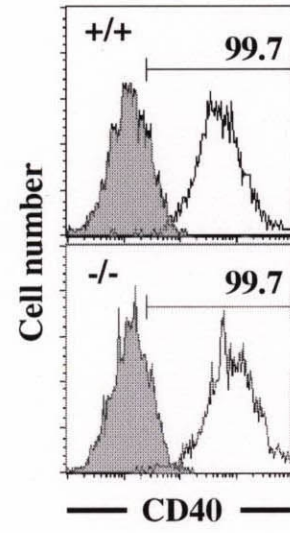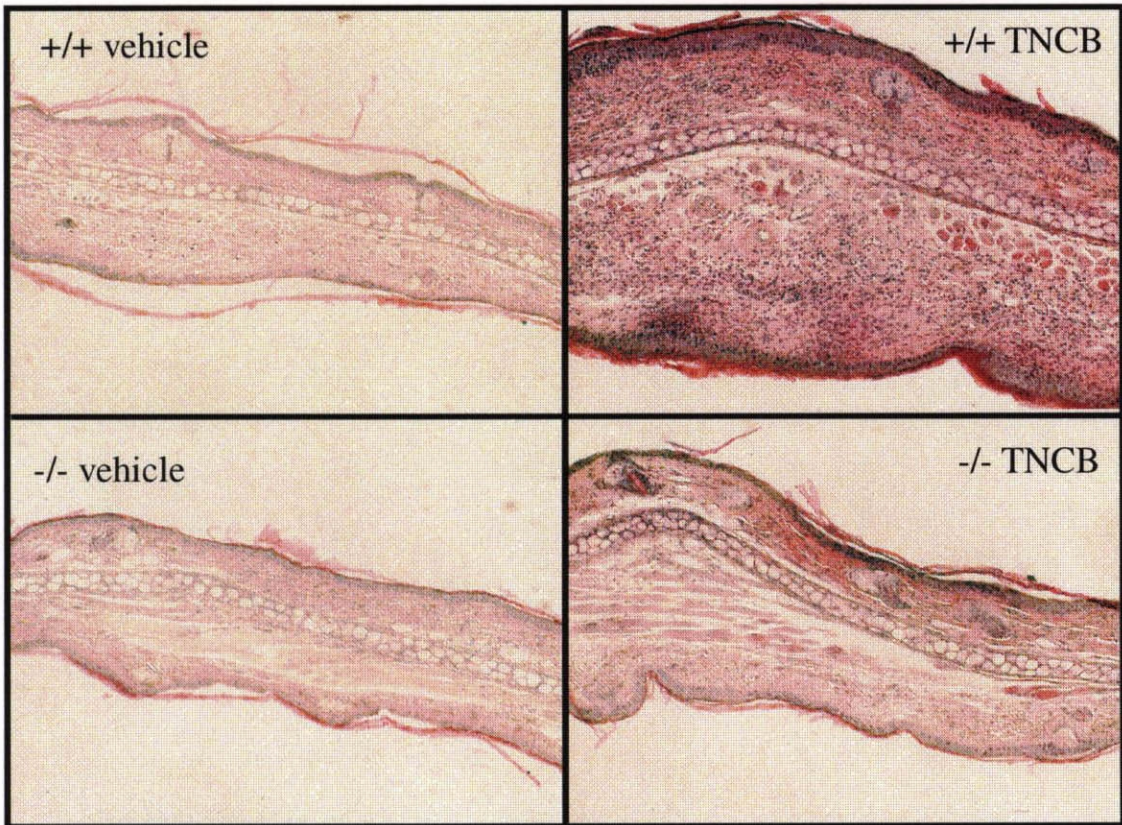
A DNFB

B TNCB

D

C

Figure 3. Impaired CHS responses in IL-17-/- mice

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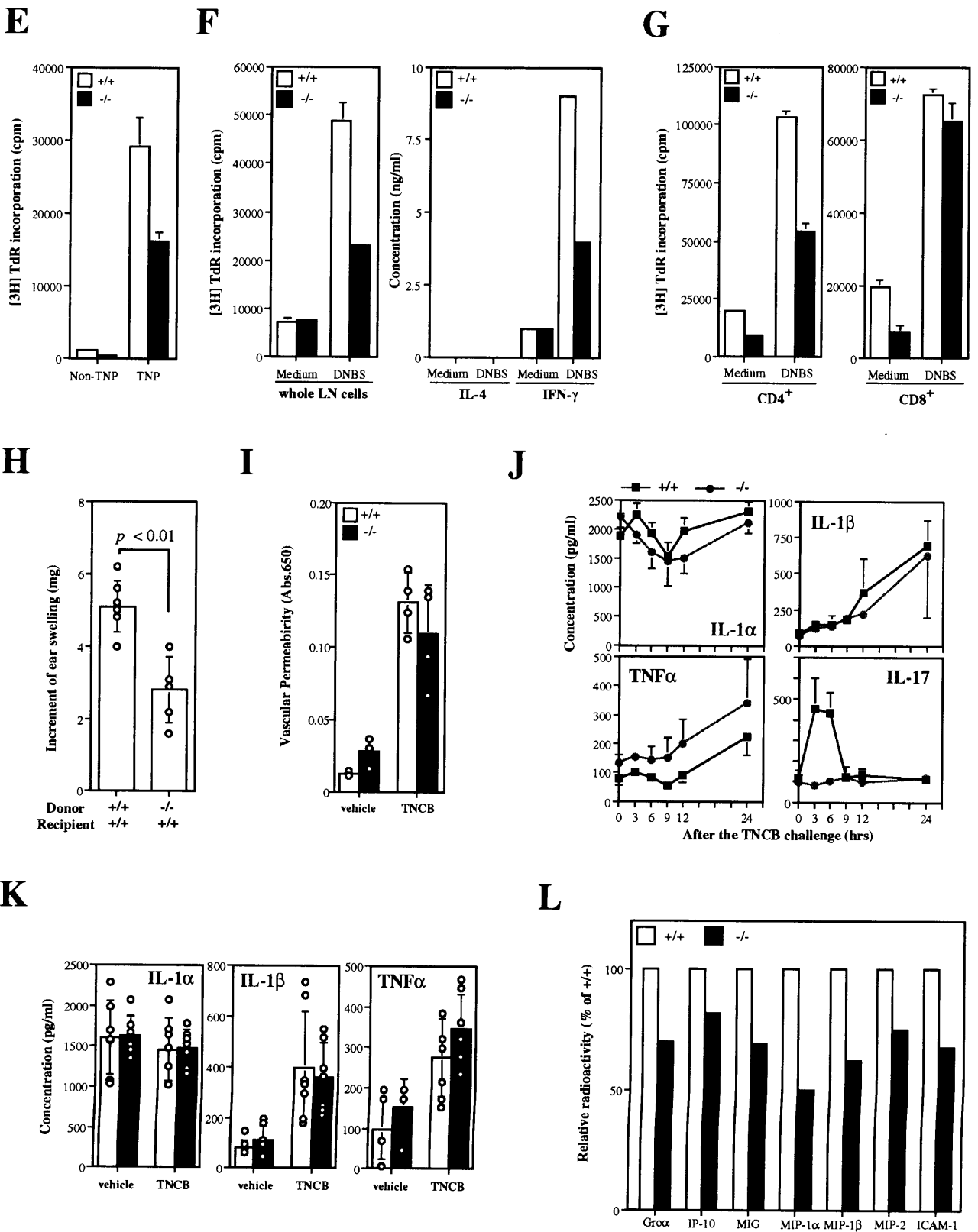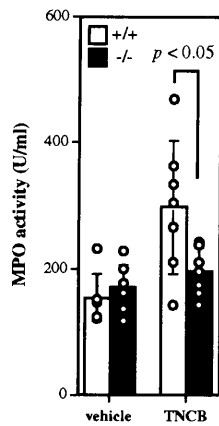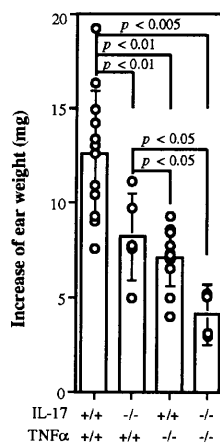
Figure 3. Impaired CHS responses in IL-17-/- mice

Figure 3. Impaired CHS responses in IL-17-/- mice

(A) Increase of ear swelling of IL-17-/- mice in DNFB-induced CHS.

(B) Increase of ear swelling of IL-17-/- mice in TNCB-induced CHS.

(C) Histological analysis of ear skin at 24h after the challenge with TNCB or vehicle alone was stained with hematoxylin-eosin.

(D) The degree of maturation of LCs migrating into draining LNs from skin painting with FITC. CD40 expression on CD11c+ FITC+ LCs was analyzed by FACS. Shaded area is shown an isotype-matched control Ig staining.

(E) TNP-specific T cell proliferation responses. Purified inguinal and axillary LN T cells from IL-17+/+ or IL-17-/- mice sensitized with TNCB were co-cultured with TNBS-treated splenic adherent cells for 3 days.

(F) 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 cultured in the absence or presence with 50 μg/ml DNBS for 3 days.

(G) Inguinal and axillary LN CD4+ or CD8+ cells from IL-17+/+ or IL-17-/- mice sensitized with DNFB were co-cultured with splenic adherent cells in the absence or presence with 50 μg/ml DNBS for 3 days.

(H) Adoptive transfer CHS response. Non-treated mice were transferred inguinal and axillary LN T cells from IL-17+/+ or IL-17-/- mice sensitized with TNCB. CHS response in T cell-transferred mice was examined by the challenge with TNCB.

(I) Vascular permeability in CHS response. Evans blue dye was injected intravenously after the challenge with TNCB, at 1 h after the challenge, exudative dye in ear skin was extracted and vascular permeability was assessed by the value of absorbance 650 nm.

(J) IL-1α, IL-1β, TNFα and IL-17 levels in the lysate of ear challenged with TNCB were measured by ELISA. Symbols were shown the average of three mice.

(K) IL-1α, IL-1β and TNFα levels in the lysate of ear challenged with TNCB after 24 hrs were measured by ELISA.

(L) Northern blot analysis of mRNA of chemokines and ICAM-1 in ear skin at 6h after the challenge with TNCB. Expression of b-actin mRNA was used to control for loaded RNA. Radio activities of chemokine mRNAs were normalized by these of β-actin mRNA, and relative values of IL-17-/- mice against IL-17+/+ mice are shown.

(M) MPO activity in CHS response. The lysate of ear skin was prepared at 24h after the challenge with TNCB and MPO activity was assessed.

(N) TNCB-induced CHS responses in IL-17-/-, TNFα-/- and IL-17-/-xTNFα -/- mice.

(A, B, H, I, K and M) Each circle represents an individual mouse, and an average and SD are shown. (E, F and G) Average ± SD of triplicate experiments is shown in proliferation assay, and cytokine levels in pooled triplicate supernatant from proliferative response assay were determined by ELISA. These results were reproducible three independent experiments.
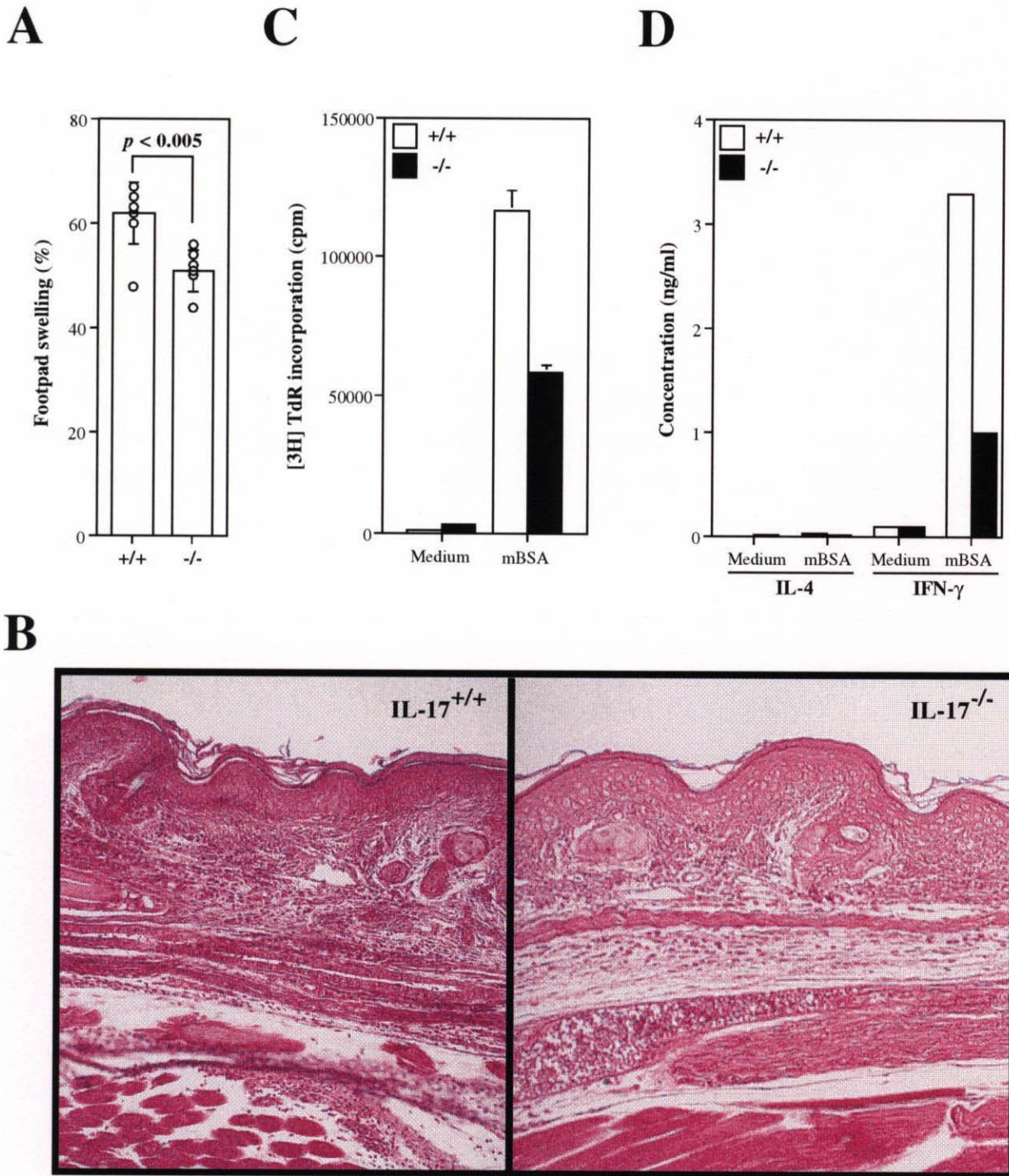
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Figure 4. Impaired DTH responses in IL-17-/- mice
(A) Increase of footpad thickness in mBSA-induced DTH response. Each circle represents an individual mouse, and an average and SD are shown. (B) Histological analysis of a footpad at 24h after the challenge with mBSA or PBS alone was stained with hematoxylin-eosin. (C) mBSA-specific T cell proliferative response. Inguinal LN cells from IL-17+/+ or IL-17-/- mice sensitized with mBSA/CFA subcutaneously were cultured in the absence or presence with 40 μg/ml mBSA for 3 days. Average ± SD of triplicate experiments is shown. These results were reproducible three independent experiments. (D) IL-4 and IFN-γ levels in pooled triplicate supernatant from proliferative response assay were determined by ELISA. These results were reproducible three independent experiments.

indicate that IL-17 also plays an important role in the induction of Th1-mediated DTH response.

AHR

IL-17 is detected in the sera of allergic asthma patients [34]. To assess whether or not IL-17 involved in the development of asthma, I analyzed OVA/Alum-induced AHR using IL-17$^{-/-}$ mice. Five days after first immunization with OVA/Alum intraperitoneally, mesenteric LN cell proliferative response of IL-17$^{-/-}$ mice against OVA was markedly reduced compared with that in IL-17$^{+/+}$ mice (Figure 5A). IL-4 and IL-5 levels in supernatants 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 comparable between the condition with and without OVA (Figure 5B). In spite of the reduced OVA-specific T cell responses, AHR to methacholine in IL-17$^{-/-}$ mice was similar levels to that in IL-17$^{+/+}$ and IL-17$^{+/-}$ mice (Figure 5C). Total infiltrated cell numbers in bronchoalveolar lavage fluids (BALF) (Figure 5D) and the pathologic morphology of the lung (data not shown) were not difference among IL-17$^{+/+}$, IL-17$^{+/-}$ and IL-17$^{-/-}$ mice sensitized with OVA/Alum. Moreover, IL-4 and IL-5 levels in BALF from IL-17$^{-/-}$ mice at 24 h after last inhalation with OVA were higher rather than those from IL-17$^{+/+}$ and IL-17$^{+/-}$ mice (data not shown). Inconsistent with these apparent observations between the reduced T cell activation and normal AHR with lung inflammation in IL-17$^{-/-}$ mice, it may be caused by the effect of Alum, which induces Th2 response independent of IL-4 and IL-13 signaling [42]. Thus, I next examine the role of IL-17 in OVA/PBS-induced AHR using IL-17$^{-/-}$xDO11.10 transgenic (Tg) mice. 24 h after last inhalation with OVA, AHR to methacholine in IL-17$^{-/-}$xDO11.10 Tg mice was partially decreased compared with that in IL-17$^{+/-}$xDO11.10 Tg mice (Figure 5E). Consistently with AHR, numbers of total infiltrated cells in BALF from IL-17$^{-/-}$xDO11.10 Tg mice was reduced compared with those from IL-17$^{+/-}$xDO11.10 Tg mice (Figure 5F). As expectedly, the lung inflammation in IL-17$^{-/-}$xDO11.10 Tg mice was also milder than that in IL-17$^{+/-}$xDO11.10 Tg mice (Figure 5G). These observations indicate that IL-17 is involved in the induction of AHR.
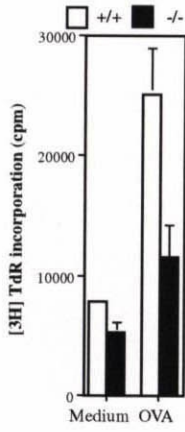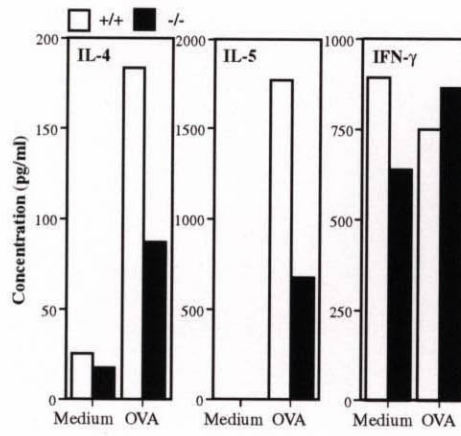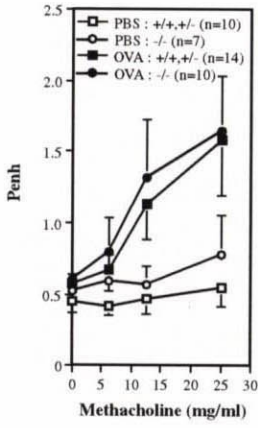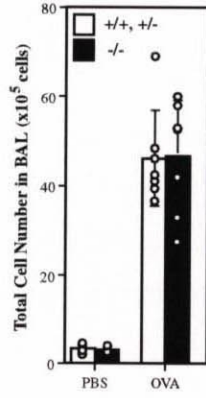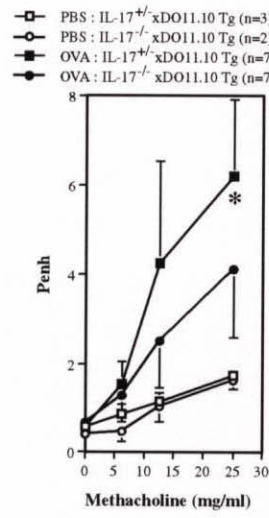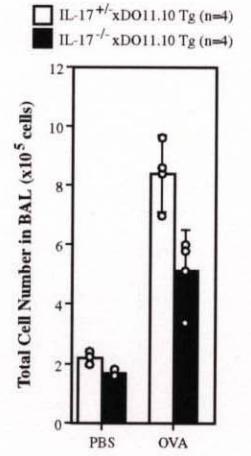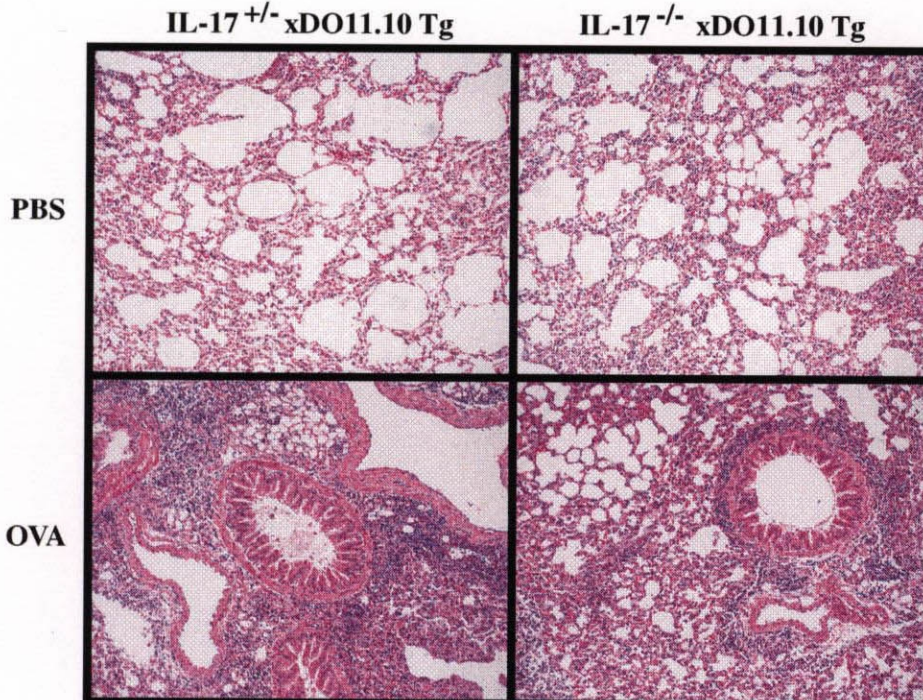
Figure 5.   AHR in IL-17$^{-/-}$ mice

(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 with 40 μg/ml OVA for 3 days.   Average ± SD of triplicate experiments is shown.   These results were reproducible three independent experiments. (B) IL-4, IL-5 and IFN-γ levels in pooled triplicate supernatant from proliferative response assay were determined by ELISA.   These results were reproducible three independent experiments. (C) AHR to aerosolized methacholine in mice sensitized with OVA/Alum intraperitoneally and challenged with OVA/PBS or PBS only intranasally.   (D) Total cell numbers in BALF.   At 24 h 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 mannually.   (E) AHR to aerosolized methacholine in IL-17$^{+/-}$ or IL-17$^{-/-}$ xDO11.10 Tg mice inhaled with OVA/PBS or PBS only intranasally.   (F) Total cell numbers in BALF.   At 24 h after the last inhalation of OVA/PBS or PBS in IL-17$^{+/-}$ or IL-17$^{-/-}$xDO11.10 Tg mice, BALF was collected and cell numbers were counted mannually as in (C).   Each circle represents an individual mouse, and an average and SD are shown.   (G) Lung histology in IL-17$^{+/-}$ or IL-17$^{-/-}$xDO11.10 Tg mice inhaled with OVA/PBS.   At 24 h after the last inhalation, the lung was collected and sections of the lung were stained with hematoxylin-eosin.   Symbols represent the average of each genotype mice with SD in (C) and (E).   Each circle represents an individual mouse, and an average and SD are shown in (D) and (F). $*p < 0.05$.

Acute GVHR

I next examined the role of IL-17 in acute GVHR. Expansion of donor IL-17$^{-/-}$ CD4$^+$ T cells (H-2K^b) in the spleen of recipient CBF1 mice (H-2K$^{b/d}$) was similar to that of IL-17$^{+/+}$ CD4$^+$ T cells (Figure 6A). Similar tendency was also observed in the expansion of the donor CD8$^+$ T cells from IL-17$^{-/-}$ mice (Figure 6A). The reduction kinetics of the recipient B cells were also similar between mice received IL-17$^{+/+}$ and IL-17$^{-/-}$ T cells (Figure 6A). Con A response of the recipient spleen cells of CBF1 mice received IL-17$^{+/+}$ and IL-17$^{-/-}$ spleen cells was reduced similarly (Figure 6B). 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 6C). These results indicate that IL-17-deficiency of donor T cells does not affect the development of acute GVHR.

T cell-dependent antibody production

Since the development of diseases in which helper T cells are involved is affected by deficiency with IL-17, effects of IL-17-deficiency on the antigen-specific T cell-dependent antibody production was studied. Antibody production is known to be affected by the route of immunization and with or without adjuvants [43,44], I investigated antibody production under different immunization conditions. In the skin sensitization route during TNCB-induced CHS response, all the level of TNP-specific IgG subclasses in sera from IL-17$^{-/-}$ mice was significantly reduced compared with that from IL-17$^{+/+}$ mice (Figure 7A). In the subcutaneous immunization with mBSA/CFA during DTH response, mBSA-specific IgG1 and IgG3 levels in IL-17$^{-/-}$ mice were markedly lower than those in IL-17$^{+/+}$ mice, and IgG2a and IgG2b levels were also reduced but not significantly (Figure 7B). Furthermore, in the intraperitoneal immunization with OVA/Alum during AHR, OVA-specific IgG1 and IgE levels in IL-17$^{-/-}$ mice were significantly reduced compared with those in IL-17$^{+/+}$ mice, while other IgG subclasses levels between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice were comparable (Figure 7C). These results indicate that IL-17 plays an important role in T-dependent antibody production.
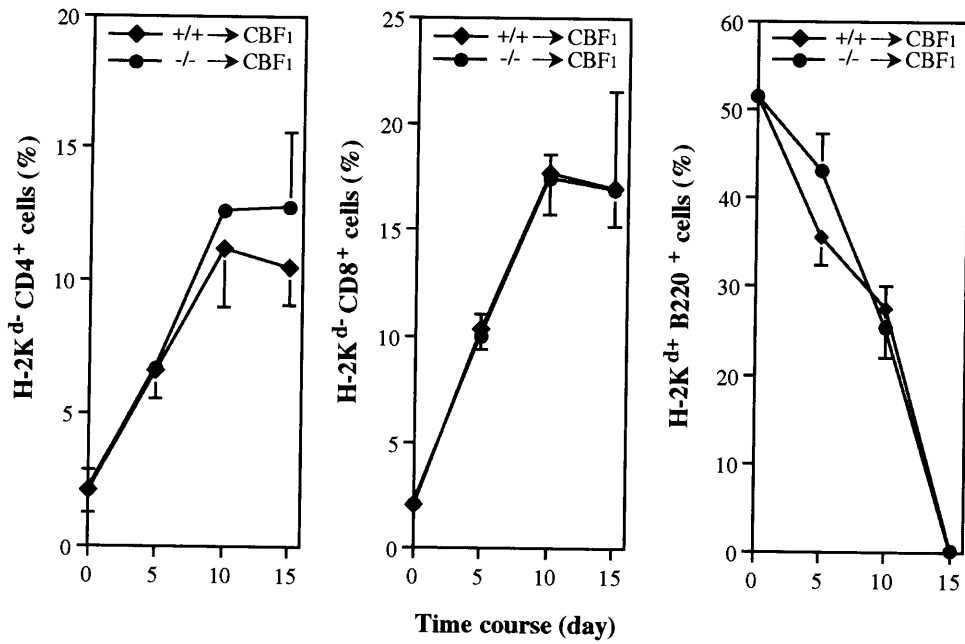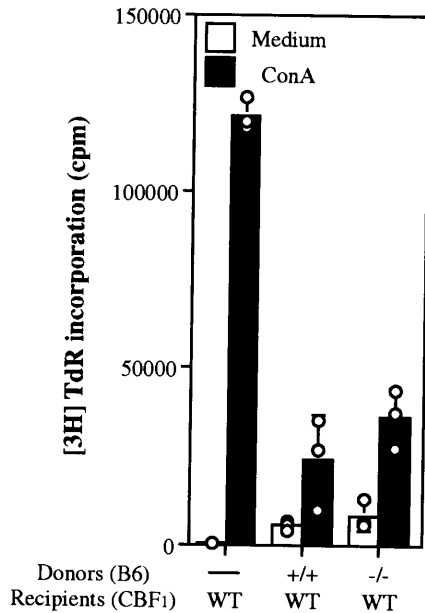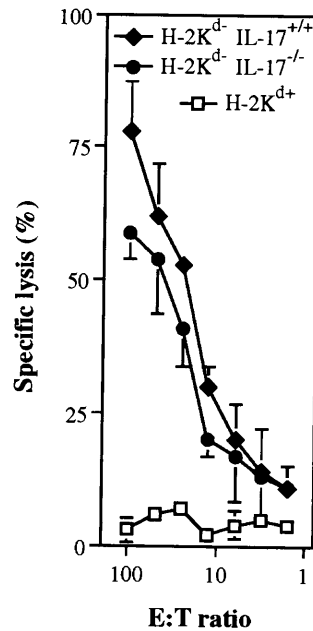
Figure 6. Nomal Acute GVHR by IL-17-/- donor cells

Spleen cells from IL-17+/+ or IL-17-/- mice on C57BL/6J background (H-2Kb/b) were transferred into CBF1 (C57BL/6J x BALB/cA F1: H-2Kb/d) mice intravenously. (A) At indicated time points after transfer, spleen of recipient CBF1 mice was harvested, and the expansion of H-2Kd-CD4+ or H-2Kd-CD8+ cells of donor IL-17+/+ or IL-17-/- spleen cells and the reduction of H-2Kd+B220+ cells of recipient CBF1 mice was analyzed by FACS. Symbols represent the average of three mice and SD. (B) Proliferative responses of recipient CBF1 spleen cells to ConA on 10 days after transfer. Each circle represents an individual mouse, and an average and SD are shown. (C) CTL activity to H-2Kd+ P815 cells. 10 days after transfer, H-2Kd-CD8+ T cells from recipient CBF1 spleen cells were purified by MACS system. Specific lysis of H-2Kd-CD8+ T cells to H-2Kd+ P815 cells was determined in a ^{51}Cr release assay. Symbols represent the average of three mice and SD.

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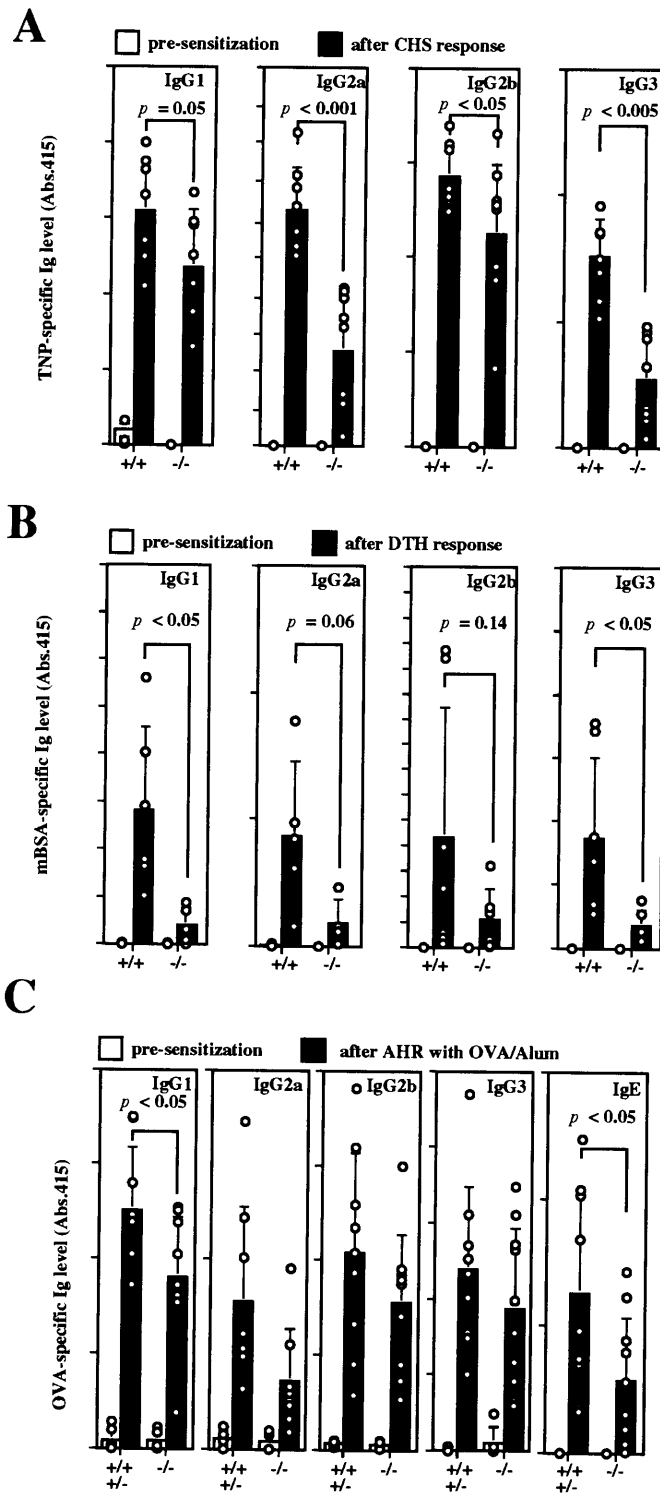
Fugure 7. A role of IL-17 in antigen-specific Ig production

(A) 3 days after the challenge with TNCB during CHS response, sera were collected.

(B) 1 week after the challenge with mBSA during DTH response, sera were collected.

(C) 24h after the last challenge with OVA during AHR, sera were collected. TNP-, mBSA- and OVA-specific Ig levels in sera were determined by ELISA. Each circle represents an individual mouse, and an average and SD are shown. A graduation of the ordinate (Abs.415) is 0.1.

121

Discussion

In the current study, I demonstrated that IL-17 plays an important role in allergen-specific T cell priming and B cell antibody production and involves in the development of allergic diseases using IL-17$^{-/-}$ mice.

Previously, it was reported that IL-17 is produced by human skin-derived nickel-specific T cells, suggesting that there is the involvement in the induction of CHS [16]. I demonstrated here that IL-17 plays an important role in the development of CHS response. CHS response is induced via various steps; migration of LC in LNs from the skin, maturation of LC and allergen-specific T cell priming through T-LC interaction in the sensitization phase and leukocyte infiltration into the site re-challenged with allergens in the elicitation phase [41]. Although it was known that IL-17 promotes maturation of DC [27], IL-17-deficiency did not affect both migration (data not shown) and maturation of LC in the sensitization phase. On the other hand, IL-17 plays important role in hapten-specific CD4$^+$, but not CD8$^+$, T cell activation. It is known that CHS response is mediated by CD8$^+$ T cells as effector cells and regulated by CD4$^+$ T cells using MHCI$^{-/-}$ and MHCII$^{-/-}$ mice [45,46]. However, in recent, both CD8$^+$ and CD4$^+$ T cells, especially Tc1 and Th1 cells, are shown to involve in the development of CHS using CD8$^{-/-}$ and CD4$^{-/-}$ mice [47]. Therefore, the reduced CHS response in IL-17$^{-/-}$ mice is caused by the insufficient hapten-specific CD4$^+$ T cell priming in the sensitization phase as shown in adoptive T cell transfer experiments.

Proinflammatory cytokines such as IL-1 and TNFα have crucial roles in induction of adhesion molecules and chemokines in various inflammatory responses. IL-17 has similar bioactivities as well as those cytokines, and IL-17 is also known to be a potent inducer of IL-1 and TNFα on human macrophages and kerationocytes [16,17,23]. In the elicitation phase, IL-1α, IL-1β and TNFα productions were normal kinetics and levels in the inflammatory sites of IL-17$^{-/-}$ mice. In our unpublished observations, IL-1 and TNFα have distinct roles in CHS responses using IL-1α/β$^{-/-}$, TNFα$^{-/-}$ and IL-1α/β$^{-/-}$ xTNFα$^{-/-}$ mice [Nakae et al., in preparation]. IL-1 as well as IL-17 is required for hapten-specific T cell priming in the sensitization phase [48], while TNFα is not [Nakae et al., in preparation]. However, both IL-1 and TNFα plays important roles in the development of inflammation in the elicitation phase [Nakae et al., in preparation]. In

this study, I showed that CHS response in IL-17$^{-/-}$xTNF$\alpha^{-/-}$ mice was lower than that in IL-17$^{-/-}$ mice or that in TNF$\alpha^{-/-}$ mice, respectively. Mice received T cells from IL-17$^{-/-}$ mice sensitized with TNCB exhibited the impaired CHS response while mice received T cells from TNF$\alpha^{-/-}$ mice developed CHS response normally [Nakae et al., in preparation]. Therefore, these results indicate that T cell-derived TNFα is not required for the development CHS and there are different roles in CHS responses between IL-17 and TNFα. In the inflammatory site during CHS responses, the peak of IL-17 protein reached earlier than IL-1β and TNFα although mRNA expression and protein levels of IL-1α were detected constitutively in the skin (data for mRNA not shown). However, both IL-1 and TNFα, but not IL-17, involve in vascular permeability in the inflammatory site at one hour after challenge with TNCB using IL-1α/$\beta^{-/-}$, TNF$\alpha^{-/-}$ and IL-1α/$\beta^{-/-}$xTNF$\alpha^{-/-}$ mice (Nakae et al., unpublished data). Moreover, the expression of mRNA for chemokines and adhesion molecules in the inflammatory site of IL-17$^{-/-}$ mice was reduced milder than that of TNF$\alpha^{-/-}$ mice or that of IL-1α/$\beta^{-/-}$ mice, respectively [Nakae et al., in preparation]. Thus, not only the specific and/or redundant roles of IL-17, IL-1α/β and TNFα but also a network of these four cytokines in the elicitation phase during CHS response still remain unknown. I will examine these questions using double- and triple-gene deficient mice of IL-17, IL-1α/β and TNFα in the future study.

AHR is mediated by Th2-dependent immune response, especially IL-4, IL-5 and IL-13 are key molecules to induce IgE production and the activation of mast cells and eosinophils [49]. AHR by sensitization with OVA/Alum in IL-17$^{-/-}$ mice was developed normally in spite of the reduced T cell responses to OVA, while that by sensitization with OVA/PBS in IL-17$^{-/-}$xDO11.10 Tg mice was decreased. In mast cell deficient mice, although OVA/Alum-induced AHR was elicited normally, OVA/PBS-induced AHR was reduced [50]. Furthermore, AHR by OVA/Alum immunization in IL-1α/$\beta^{-/-}$ mice as well as in mast cell-deficient mice exhibited normally while that by OVA/PBS was significantly reduced (Nakae et al., unpublished data). In the case for this notion, the alum induces Th2 response independent of IL-4 and IL-13 signaling, suggesting that the sensitization with antigen mixed with the alum can promote immune cells strongly but has a non-specific feature [42]. Therefore, IL-17 has an important role in the development of allergic airway hypersensitivity through the allergen-specific T cell

activation without the alum.

DTH response is an IFN-γ-producing Th1 cell-mediated cellular immune response, which is different from CHS response [41]. Both DTH and AHR in IL-17$^{-/-}$ mice were reduced, and IL-17 is required for allergen-specific T cell priming in the sensitization phase during DTH and AHR as well as during CHS response. Although it is reported that IL-17 is produced by Th0/Th1, but not by Th2 cell clones established from rheumatoid arthritis patients, IL-17 produced by both Th1 and Th2 cell clones from human skin-derived nickel-specific T cells [15,16]. Therefore, T cells producing IL-17 may not be restricted the classification of Th1/Th2, since it was reported that IL-17 is produced by T cells expressing TNFα, not by Th1 or Th2 cells in mouse and is not essential for Th1/Th2 differentiation [14]. IL-17 may be two-sided property in both Th1 and Th2 response as IL-18, which augment IFN-γ production synergistically with IL-12 in Th1 response [51] and IL-4-dependent IgE production in Th2 response [52], although the molecular mechanism by IL-17 remains still unknown in detail.

In host-versus-graft rejection (HVGR) such as allograft transplantation, which is a T cell-mediated immune response, although it is known that the blockade of IL-17-IL-17R binding by IL-17R:Fc treatment resulted in prolonged allograft survival [27], a role of IL-17 in acute GVHR is still unknown. However, IL-17-deficiency of donor T cells did not affect the rejection of recipient B cells, and alloantigen-specific CTL activity of IL-17$^{-/-}$ CD8$^+$ T cells was also shown normally. It was reported that expansion of allo-specific CD8$^+$ T cell in short term of acute GVHR occurred without CD4$^+$ T cells and also known that there was induction of cytotoxic CD8$^+$ T cells to alloantigen is not only dependent but also independent of CD4$^+$ T cells [53,54]. Therefore, in contrast to CHS, IL-17 from CD4$^+$ T cells is not required for allo-specific CD8$^+$ T cell activation in acute GVHR.

Antigen-specific Ig production in the humoral immunity is essential for host defense as well as the cellular immunity. In mouse models, antibody production is known to be affected by the immunization protocols; a kind of antigens (SRBC as particle antigens and KLH and OVA as soluble protein antigens) with or without adjuvants, a kind of adjuvants (IFA, CFA and Alum) and an immunization route (intraveneous, intraperitoneal, subcutaneous and intradermal administration) [43,44,55,56]. IL-17-deficiency did not have a direct effect on B cell function to non-specific

mitogenic stimuli such as LPS, anti-IgM mAb and anti-CD40 mAb (data not shown). However, antigen-specific B cell antibody production in IL-17$^{-/-}$ mice was significantly decreased during CHS response via skin without an adjuvant, during DTH response in the immunization intraperitoneally with CFA and during AHR in the immunization intraperitoneally with Alum, suggesting that the impaired antigen-specific T cell activation in IL-17$^{-/-}$ mice resulted in insufficient B cell responses.

Taken all together, these results suggest that IL-17 has an important role in the development of allergen-specific T cell-mediated responses through the activation of both cellular and humoral immunities. Our findings provide a novel insight into molecular mechanisms of development of CHS, DTH and AHR. IL-17 is implicated to involve in the other T cell-mediated inflammatory responses such as collagen-induced arthritis, inflammatory bowel disease and experimental autoimmune encephalomyelitis. Using IL-17$^{-/-}$ mice, further elucidation of the control mechanisms of IL-17 should provide us with important cues in the quest to develop therapeutics for these diseases.

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DISCUSSION AND CONCLUSION

In the present study, I investigated the roles of IL-1α, IL-1β, TNFα and IL-17 in T cell-mediated immune responses using these gene-deficient mice. I showed here that these cytokines play important roles in both humoral and cellular immunities and have distinct and/or redundant activities in several responses.

IL-1 has bipolar properties as a T cell co-stimulatory factor in the sensitization phase and as a proinflammatory cytokine in the elicitation phase during CHS response, DTH response (Nambu et al., in preparation) and AHR (Nakae et al., in preparation)[31]. And then, IL-1 promotes antigen-specific antibody production of B cells through the activation of antigen-specific T cells. IL-1 is also responsible for the development of autoimmune responses such as collagen-induced arthritis (CIA) by the activation of T cells to autoantigens (Saijo et al., in press). Moreover, this cytokine is implicated to induce another autoimmune responses, experimental autoimmune encephalomyelitis (EAE) although the mechanism by IL-1 still remains unknown [32].

IL-1 was discovered as a growth factor of thymocyte in 1970s [6], and it is known that IL-1 promotes proliferative response of T cells to Con A [6]. However, in those days, IL-1 which researchers used in their assays was very crude. Therefore, some old findings do not always show the correct activities of IL-1 in immune responses including the effect of IL-1 on T cell activation, since CD28-CD80/CD86 co-signaling was demonstrated to be a major pathway to activate T cells. However, I demonstrated here that IL-1 is required for antigen-specific T cell priming independent of CD28-

132

CD80/CD86 co-signaling and for the induction of the other T cell co-stimulatory molecules, CD40L and OX40. CD40L and OX40 as well as CD28 play important roles in various T cell-mediated immune responses including CHS, DTH, AHR, CIA, EAE, inflammatory bowel diseases (IBD), acute and chronic graft-versus-host diseases (GVHD), host-versus-graft diseases (HVGD) and diabetes, suggesting that IL-1 involves in the development of these responses by the activation of T cells and other immune cells dependent of CD40L-CD40 and OX40-OX40L [33-53]. Therefore, these findings should provide us the understanding of molecular mechanisms in the development of various immune responses by IL-1.

IL-1 is regarded as a major proinflammatory cytokine rather than as a T cell-costimulatory molecule. Indeed, the administration of rIL-1 resulted in local inflammation with neutrophil infiltration [6]. Recently, it was shown that IL-1 can induce various adhesion molecules and chemokines to recruit neutrophils and macrophages into inflammatory site [54]. It was shown that transgenic (Tg) mice overexpressing IL-1α and IL-1RI specifically on keratinocytes developed spontaneous skin inflammation with neutrophil infiltration [55,56] and PMA-induced skin inflammation in IL-1RII Tg mice was markedly suppressed [57]. Mice lacking a IL-1Ra gene (IL-1Ra$^{-/-}$ mice) and overexpressing human IL-1α under β-actin promoter developed spontaneous chronic inflammatory arthropathy resembling rheumatoid arthritis with the infiltration of neutrophils and macrophages, respectively [58,59]. These observations indicate strongly that IL-1 is responsible for the induction of local inflammation. However, it was reported that CHS with local epidermal inflammation in IL-1β$^{-/-}$ mice

occurred normally [25] and that DTH with local dermal inflammation in IL-1RI$^{-/-}$ mice was significantly suppressed, but not completely [29]. These results implicate that the other proinflammatory cytokines such as TNFα compensate IL-1 activities, since TNFα has similar pathological activities to IL-1 in various host defense responses such as fever development, endotoxin shock and acute phase responses [6]. In the support for this notion, neither TNFα nor IL-1 are shown to be essential for the inflammatory response in LPS-induced airway diseases in the study using TNFα$^{-/-}$ and IL-1RI$^{-/-}$ mice [60]. It is suggested that TNFα in IL-1RI$^{-/-}$ mice and IL-1 in TNFα$^{-/-}$ mice compensate their deficiency each other. On the other hand, although IL-1 and TNFα can induce them mutually, TNFα play a significant role in LPS-induced toxicity while IL-1 does not (Asano et al., in preparation) [23,28], suggesting that the induction of IL-1-independent TNFα is responsible for this case. On the contrary to LPS-induced endotoxin shock, it was found that IL-1 rather than TNFα have a pivotal role in LPS-induced uveitis in the study using TNFRI$^{-/-}$, TNFRII$^{-/-}$, TNFRI/II$^{-/-}$, IL-1RI$^{-/-}$ and IL-1RI$^{-/-}$xTNFRI$^{-/-}$ mice although the apparent difference of inflammation mechanisms between LPS-induced airway diseases and LPS-induced uveitis was unclear [61]. Taken together, it is suggested that there is the induction of inflammation dependent of both IL-1 and TNFα signaling and/or dependent of only one of them in innate immunity for LPS and the specific and/or overlapping roles between IL-1 and TNFα.

In my studies, these findings also applied to the roles of IL-1 and TNFα in an acquired immune response such as CHS. I showed clearly that both IL-1 and TNFα are required for the induction of local inflammation, especially, IL-1-dependent TNFα

is a potent mediator in this response.   Interestingly, TNFα is in a downstream pathway

to IL-1 in the elicitation phase during CHS response as shown in Chapter IV, while

TNFα is the first secreted cytokine and induces IL-1 and IL-6 in acute phase responses [2].

This difference of the relation of IL-1 and TNFα between CHS and acute phase

responses may be explained for the constitutive expression of IL-1α mRNA and protein

on the skin while TNFα expression does not.

I showed here that there is the distinct role between IL-1 and TNFα in T cell

activation.   TNFα as well as IL-1 has been known to be a lymphocyte activator [62],

however, TNFα does not have an effect on antigen-specific T cell activation in the

sensitization phase during CHS response.   In the support for this notion, similar

observation was reported in mice lacking AUUUA sequence, which involves in mRNA

stability, of TNFα mRNA, TNF$^{\Delta ARE/\Delta ARE}$ mice [63].   Since this mutation resulted in the

excess TNFα signaling in a body, these mutant mice not only developed arthritis as well

as IL-1Ra$^{-/-}$ mice and IL-1α Tg mice but also occurred inflammatory bowel diseases

spontaneously.   Moreover, it was shown that the spontaneous arthritis could not be

suppressed in TNF$^{\Delta ARE/\Delta ARE}$ x rag2$^{-/-}$ mice, who do not have T cells [63].   In another

autoimmune model, the development of EAE delayed in TNFα$^{-/-}$ mice, however, MOG-

specific T cell responses in these mutant mice occurred normally [64].   Therefore, my

finding and these observations indicate that TNFα is not essential for T cell priming

with antigens although it has a crucial role in the induction of local inflammation.

IL-17 has been discovered as a T cell co-stimulatory molecule in 1990s.   The

administration of rIL-17 resulted in the local infiltration of neutrophils as well as that of

IL-1 and TNFα [65], and can induce their cytokines on macrophages [66]. Therefore, IL-17 is regarded as a proinflammatory cytokine. Interestingly, IL-17 is produced restrictedly by activated and memory CD4$^+$ T cells [67], whereas IL-1 and TNFα are produced by various cells such as macrophages, fibroblasts and epithelial and endothelial cells [2]. This finding implicates that IL-17 involves in T cell-dependent inflammatory responses. Indeed, I showed that IL-17 was responsible for T cell-mediated immune responses such as CHS, DTH, AHR and antibody production using IL-17$^{-/-}$ mice. Moreover, IL-17 has a different role from TNFα in CHS induction although IL-17 is known to express on T cells producing TNFα. On the other hand, IL-17 is required for T cell priming with antigens like activities with IL-1. Interestingly, DNP-specific T cell proliferation and IL-17 production in IL-1α/β$^{-/-}$ mice sensitized with DNFB was markedly reduced compared with those from wild-type mice, those in IL-1Ra$^{-/-}$ mice was greatly enhanced and those in TNFα$^{-/-}$ mice was comparable (Figure. 1A and B). These results suggest that IL-1 produced by APCs involves in T cell priming by IL-17 production on CD4$^+$ T cells directly or indirectly. It is possible that the crosslinking between OX40 on CD4$^+$ T cells and OX40L on APCs may be a key signal in the indirect production of IL-17 by IL-1, since OX40 signal induces various cytokine productions on CD4$^+$ T cells. IL-1 is a potent inducer of CD40L, which induces OX40L on APCs, and OX40 expressions on CD4$^+$ T cells as shown in Chapter I. In fact, consistently with proliferative responses, OX40 expression on CD4$^+$ T cells in DNP-specific LN cell culture was reduced in IL-1α/β$^{-/-}$ mice, was enhanced in IL-1Ra$^{-/-}$ mice and was comparable in TNFα$^{-/-}$ mice (Figure. 1C). On the contrary to this hypothesis, IL-17 induced by IL-1 is possible to

promote OX40 expression on CD4$^+$ T cells. Thus, I think three hypotheses in antigen-specific T cell priming by IL-1 and IL-17; 1) OX40 expression by IL-17, 2) IL-17 production by OX40 and 3) independent stimulation by IL-17 and OX40. I postulated this process as shown in Figure 2.

I could not shown whether or not IL-17 derived from T cells is essential for the induction of the local inflammation, because IL-17 affects T cell priming in the sensitization phase such as CHS, DTH and AHR. I showed that the mRNA expression of various chemokines and ICAM-1 in the local inflammatory sites during CHS response was reduced in IL-17$^{-/-}$ mice in spite of normal expression of IL-1α/β and TNFα mRNAs. However, these reduced mRNA expression may be caused by the insufficient antigen-specific T cell activation. To determine the role of IL-17 derived from T cells in the local inflammation, I should induce these responses in IL-17R$^{-/-}$ mice received T cells of wild-type mice sensitized with antigens or treat with anti-IL-17 antibody or soluble IL-17R to block local IL-17 activities in mice after challenge with antigens.

In summary, the results presented in this study indicate the distinct roles of IL-1α, IL-1β, TNFα and IL-17 in the T cell-mediated immune responses. I believe that this study will contribute not only to understand the fundamental molecular mechanism of immune response but also to find the therapy of autoimmune diseases by the failure of immune system such as rheumatoid arthritis, ulcerative colitis, systemic lupus erythematosus, psoriasis, lichen sclerosus, alopecia areata and Sjogren's syndrome.
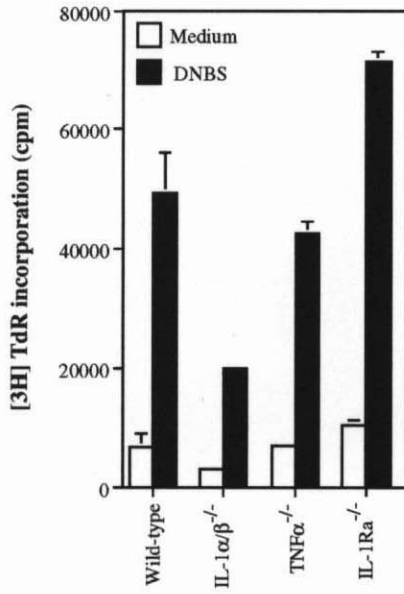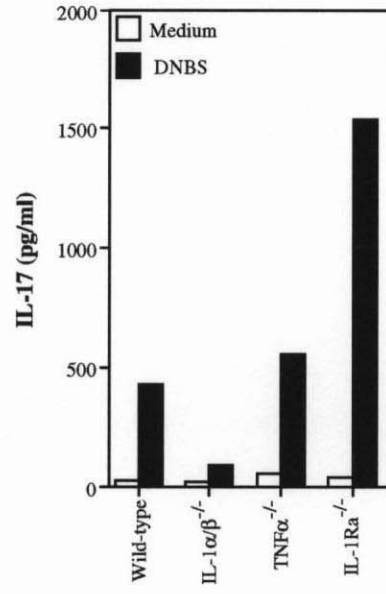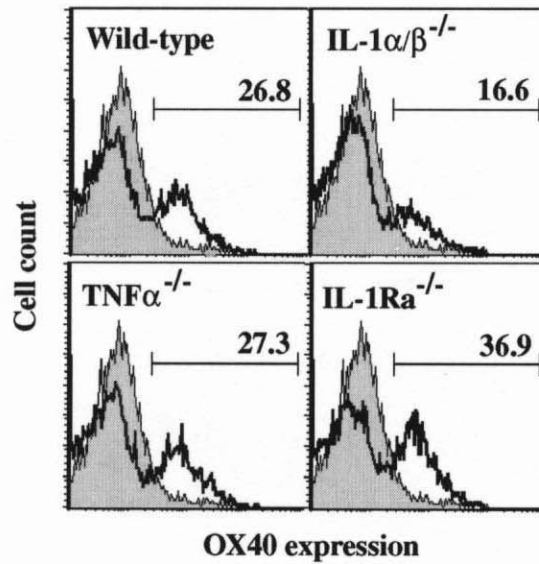
**Figure 1 DNP-specific T cell responses in IL-1α/β-/-, IL-1Ra-/- and TNFα-/- mice**

The abdomen of mice was shaved and sensitized epicutaneously with 50 ml of 0.5% DNFB.
On day 5 after sensitization, inguinal and axillary lymph nodes were harvested and single cell
suspensions were prepared. Lymph node cells of IL-1α/β-/-, IL-1Ra-/- and TNFα-/- mice were
cultured in the absence or presence with 50 ml/ml DNBS for 72 h.

(A) Proliferative response to DNBS was assessed by the incorporation of [3H]-thymidine.
Average ± SD of triplicate experiments is shown. One representative result from three independent
experiments is shown.

(B) IL-17 levels in the supernatants of DNP-specific lymph node cell culture were determined by
ELISA. One representative result from three independent experiments is shown.

(C) The expression levels of OX40 on CD4+ T cells were analyzed by FACS under the condition
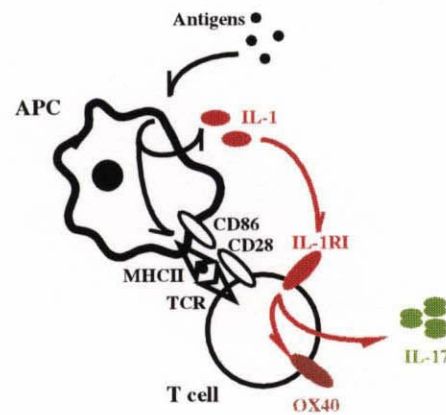 of (A).

138
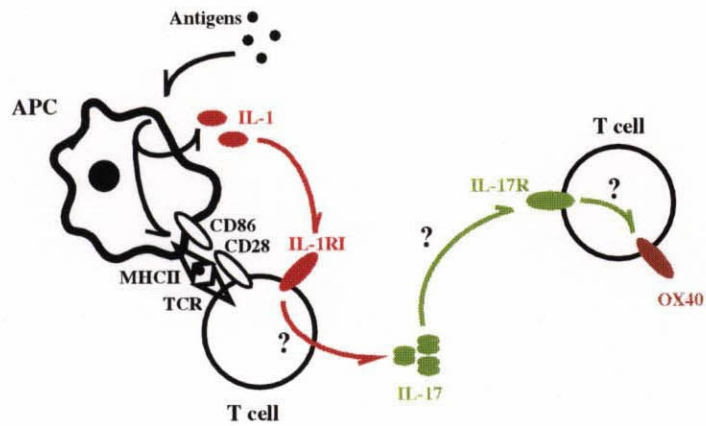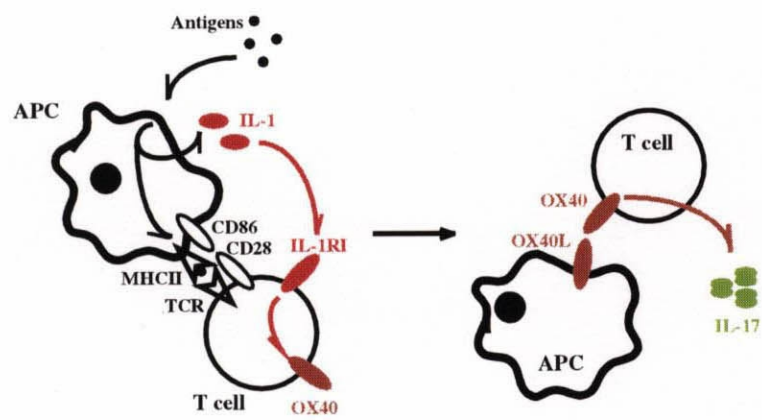
Figure 2 The molecular mechanism in T cell priming

IL-1 produced by antigen-presenting cells (APCs) binds IL-1RI on T cells.

(A) IL-1 induces IL-17 by T cells, and then, IL-17 promotes OX40 expression on T cells (hypothesis 1).

(B) IL-17 is produced by T cells through the crosslinking of OX40 induced by IL-1 on T cells and OX40L on APCs (hypothesis 2).

(C) IL-1 induces IL-17 production and OX40 expression on T cells directly(hypothesis 3).

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