Studies on Roles of Interleukin-1, Interleukin-17 and Tumor Necrosis Factorα in T Cell-Mediated Immune Responses

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CONTENTS

ABBREVIATIONS USED

Ab, antibody
Abs., absorbancy
AHR, airway hypersensitivity response
AP, alkaline phosphatase
APC, antigen presenting cell
BALF, bronchoaveolar lavage fluid
BSA, bovine serum albumin
C, complement
CCR, CC chemokine receptor
CD, cluster of differentiation
CD40L, CD40 ligand
CFA, complete Freund's adjuvant
CHS, contact hypersensitivity
ConA, concanavalin A
cox, cyclooxygenase
CTL, cytotoxic T lymphocyte
CTLA, cytotoxic T lymphocyte associated antigen
CXCR, CXC chemokine receptor
DC, dendritic cell
DNCB, 2,4-dinitrochlorobenzene
DNFB, 2,4-dinitrofluorobenzene
DNP, dinitrophenyl
DT, diphtheria toxin A
DTH, delayed-type hypersensitivity
EBV, Epstein-Barr virus
EC, epidermal cell
EGFP, enhanced green fluorescent protein
ELC, EBV-induced molecule 1 ligand
ELISA, enzyme-linked immunosorbent assay
ES, embryonic stem
FcγR, Fcg receptor
FCS, fetal calf serum
FDC, follicular dendritic cell
FITC, fluorescein isothiocyanate
G-CSF, granulocyte-colony stimulating factor
GM-CSF, granulocyte-macrophage colony-stimulating factor
Gro, growth-regulated oncogene

GVHR, graft-versus-host reaction
HRP, horseradish peroxidase
HVGR, host-versus-graft reaction
HVS, Herpesvirus saimili
I-TAC, IFN-inducible T cell alpha chemoattractant
ICAM-1, intracellular adhesion molecule-1
IFA, incomplete Freund's adjuvant
IFN-g, interferon-gamma
Ig, immunoglobulin
IL, interleukin
IL-17R, IL-17 receptor
IL-1Ra, IL-1 receptor antagonist
IL-1RI, IL-1 receptor type I
IL-1RII, IL-1 receptor type II
IL-2Ra, IL-2 receptor alpha chain
iNOS, inducible nitric oxide synthetase
IP-10, IFN-γ-inducible protein 10 kDa
KC, keratinocyte
KLH, keyhole lympet hemocyanin
L. major, *Leishmania major*
LC, Langerhans cell
LCMV, lymphocytic choriomeningitis virus
LN, lymph node
LPS, lipopolysaccharide
mAb, monoclonal antibody
mBSA, methylated bovine serum albumin
MCP, monocyte chemoattractant protein
MHC, major histocompatibility complex
MIG, monokine induced by IFN-gamma
MIP, macrophage inflammatorypProtein
MPO, myeloperoxidase
Nb, *Nippostrongylus brasiliensis*
NK, natural killer
NP-CGG, nitrophenyl-conjugated chicken gamma globulin
NP-OVA, nitrophenyl-ovalbumin
ODF, osteoclast differentiation factor
ORF, open reading frame
OVA, ovalbumin
OX40L, OX40 ligand

PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PEC, peritoneal exudate cell
PerCP, peridinin chlorophyl-a protein
PG, prostaglandin
PGK, phosphoglycerate kinase
PHA, phytohemagglutinin
PMA, phorbol 12-myristate 13-acetate
PMN, polymorphonuclear leukocyte
r, recombinant
RANTES, regulated upon activation, normal T expressed and secreted
SAC, splenic adherent cell
SCF, stem cell factor
SD, standard deviation
SLC, secondary lynphoid organ chemokine
SRBC, sheep red blood cell
TBS, Tris-buffered saline
TCR, T cell receptor
TD, T-dependent
Tg, transgenic
TGC, thioglycolate
Th, T helper
TI, T-independent
TMEV, Theiler's murine encephalomyelitis virus
TNBS, trinitrobenzene sulfonate
TNCB, 2, 4, 6-trinitrochlorobenzene
TNF, tumor necrosis factor
TNFRI, TNF receptor type I
TNFRII, TNF receptor type II
TNP, trinitrophenyl
TRAF, TNF-associated factor
VSV, vesicular stomatitis virus

INTRODUCTION

A cytokine, a small molecular weight glycoprotein produced by various cells, is a major mediator of host defense responses against environmental antigens and stresses through the activation of immune system.

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are major proinflammatory cytokines produced by various cells such as macrophages and epithelial and endothelial cells during acute and chronic inflammatory responses [1,2]. It is recently shown that IL-1/IL-1 receptor (IL-1R) and TNF/TNF receptor (TNFR) form large families (Figure 1 and 2) [3-5]. Especially, IL-1α, IL-1β and TNFα in these families are necessary for the inflammation to remove invaded antigens and to recover the damaged tissue through the regulation of immune, endocrine and nervous systems [6,7]. On the other hand, these three proinflammatory cytokines have partly overlapped activities in these responses. Functional redundancy among IL-1α, IL-1β and TNFα could be caused by their partially overlapped signal transduction cascades (Figure 3) and induction of the same transcription factors such as NF-κB and AP-1[8-11].

Moreover, IL-17, which is produced by T cells, has been recently discovered as a proinflammatory cytokine [12]. IL-17 and IL-17R also form the IL-17 and IL-17R families, respectively (Figure 4) [13,14] [15-20]. It is known that biological activities of IL-17 are similar to those of IL-1α/β and TNFα in inflammatory responses. Interestingly, the signal transduction of IL-17-IL-17R is mediated by TNF-associated factor 6 (TRAF6), which is also a mediator of IL-1 signaling (Figure 3) [21].

In 1990s, IL-1α/β-, TNFα- and their receptor-deficient mice have been generated, and the importance of their cytokines in the development of fever and endotoxin shock during acute inflammatory responses has been elucidated [22-30]. However, the molecular mechanism of these cytokines in the activation of innate and acquired immune system still remains to be elucidated. To understand the specific and/or redundant activities of IL-1α, IL-1β and TNFα in physiological and pathological conditions, not only each single gene-deficient mice but also double- or triple-gene deficient mice are necessary to be examined. Furthermore, physiological and pathological function of IL-17 in a body has been largely unknown, because mice lacking of the IL-17 gene have not been generated yet.

In this current study, I have investigated the specific and/or synergistic roles of IL-1α, IL-1β, TNFα and IL-17 in the inflammatory responses through the activation of immune system in a body using these gene-deficient mice. In Chapter I and II, I studied the role of IL-1 in antibody production and T cell priming using IL-1α-, IL-1β, IL-1α/β- and IL-1 receptor antagonist (IL-1Ra)-deficient mice. In Chapter III and IV, I showed the distinct roles of IL-1α, IL-1β and TNFα in contact hypersensitivity response using IL-1α-, IL-1β-, IL-1α/β-, TNFα- and IL-1α/βxTNFα-deficient mice. In Chapter V, I described generation of IL-17-deficient mice and the role of IL-17 in T cell-mediated inflammatory immune responses.

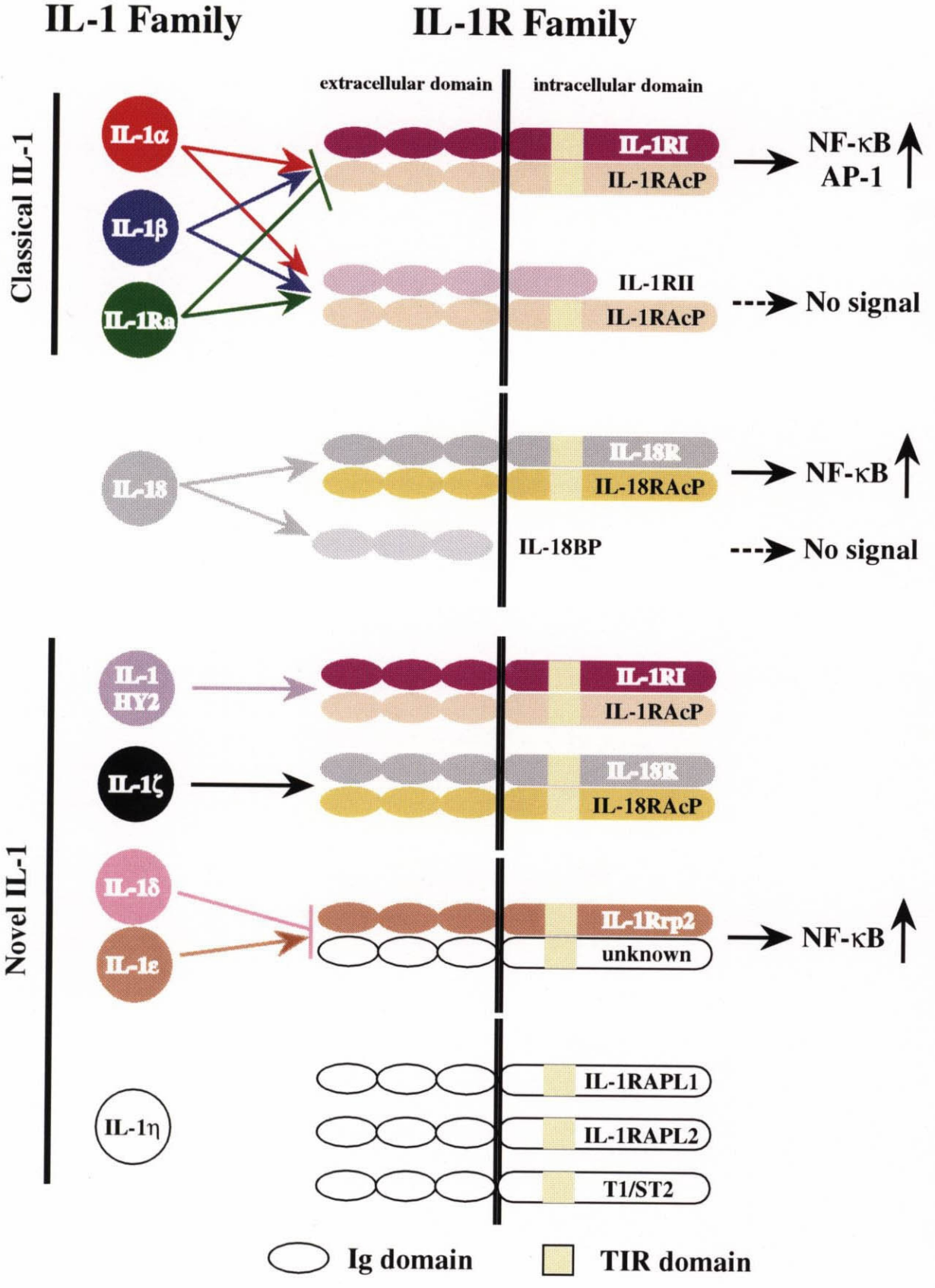

Figure. 1 IL-1 and IL-1R Families

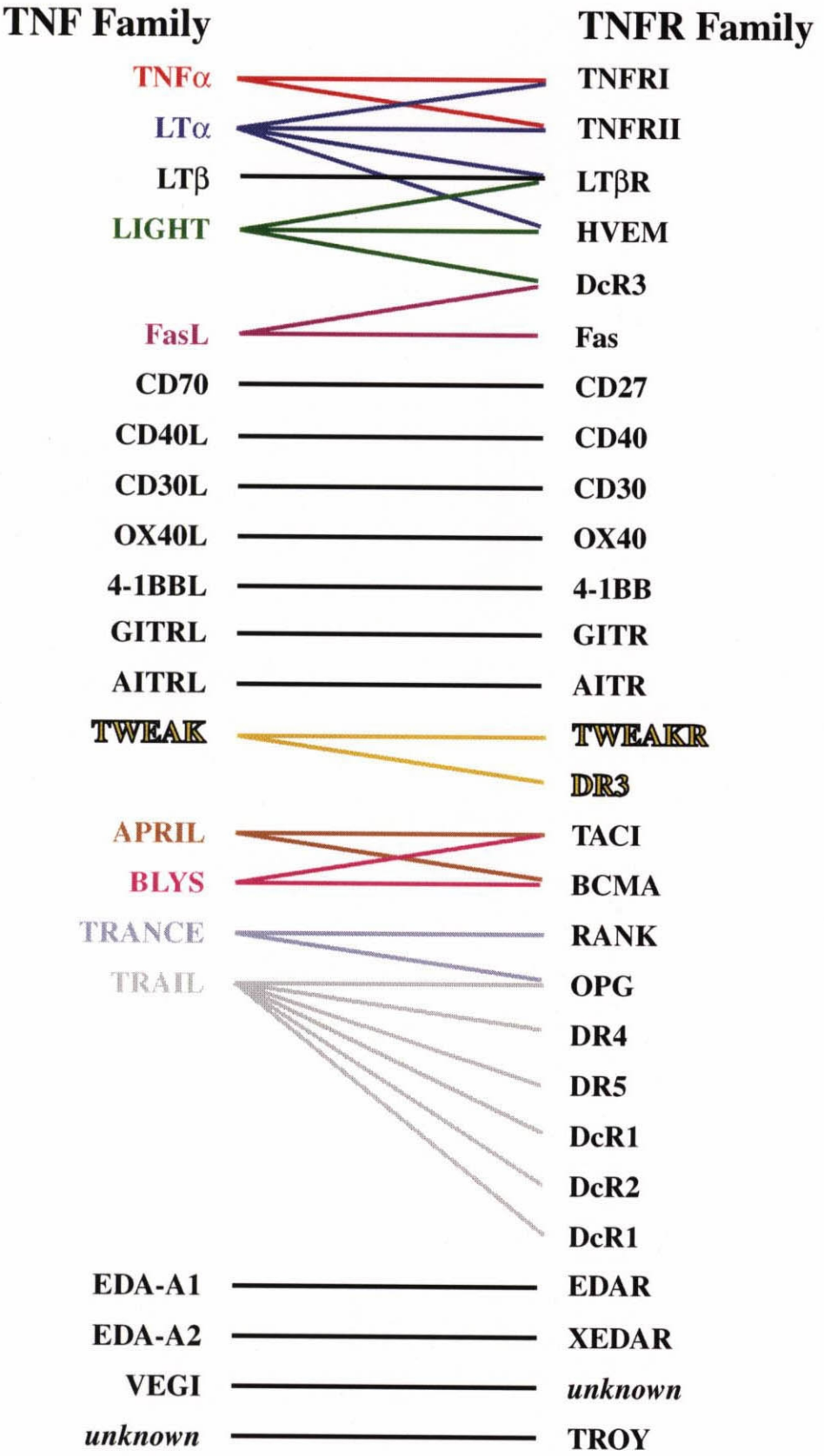

Figure. 2 TNF and TNFR Families

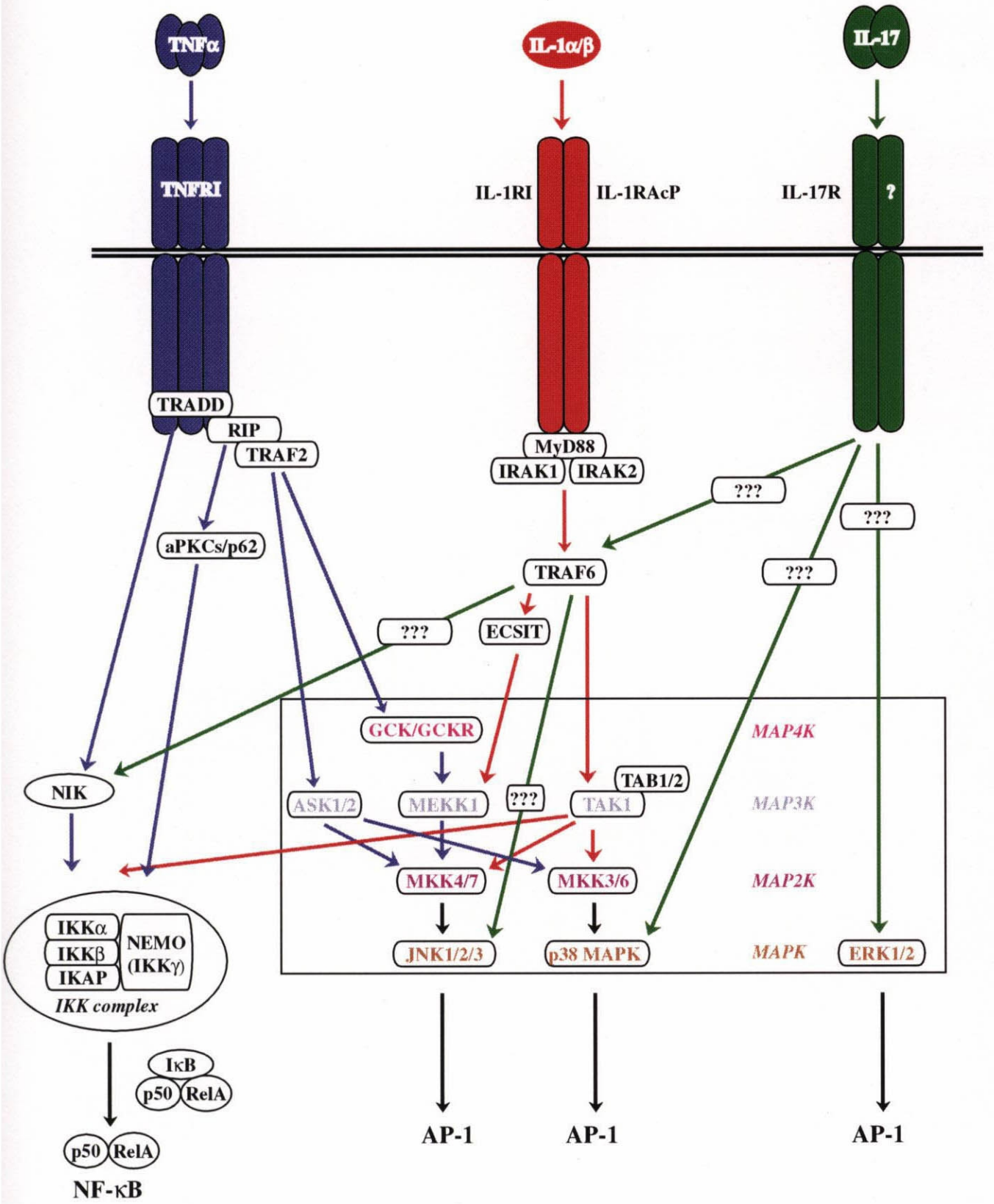

Figure. 3 Signal Transduction Cascades of IL-1α/β, TNFα and IL-17

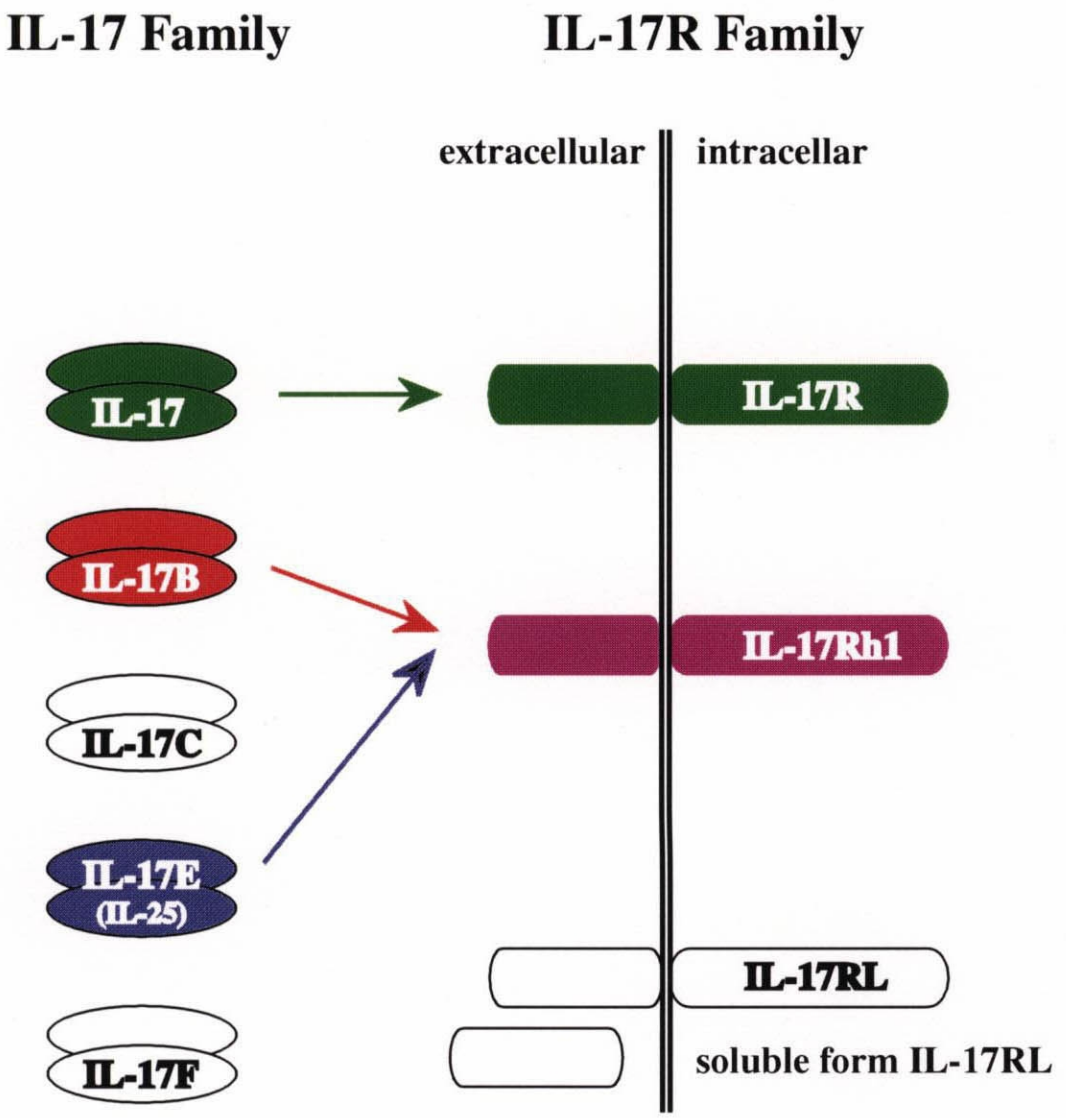

Figure. 4 IL-17 and IL-17R Families

CHAPTER I

IL-1 Enhances T cell-dependent Antibody Production through Induction of CD40L and OX40 on T Cells

Summary

IL-1 is a proinflammatory cytokine that plays pleiotropic roles in host defense mechanisms. I investigated the role of IL-1 in the humoral immune response using gene-targeted mice. Ab production against SRBC was significantly reduced in IL-1α/β-deficient ($IL\text{-}1^{-/-}$) mice, and enhanced in IL-1 receptor antagonist $(IL\text{-}1Ra)^{-/-}$ mice. The intrinsic functions of T, B, and APCs were normal in $IL\text{-}1^{-/-}$ mice. However, I showed that IL-1 $^{-/-}$ APCs did not fully activate DO11.10 T cells, while $IL\text{-}1Ra^{-/-}$ APCs enhanced the reaction, indicating IL-1 promotes T cell-priming through T-APC interaction. The function of IL-1 was CD28-CD80/CD86-independent. I found that CD40L and OX40 expression on T cells were affected by the mutation, and the reduced antigen-specific B cell response in $IL\text{-}1^{-/-}$ mice was recovered by the treatment with agonistic anti-CD40 mAb both *in vitro* and *in vivo*. These observations indicate that IL-1 enhances T cell-dependent Ab production by augmenting CD40L and OX40 expression on T cells.

Introduction

Although IL-1 was first discovered as a major mediator of inflammation, it has gradually become evident that this cytokine has numerous functions related to host defense mechanisms, not only regulating the immune system, but also the areas of the neuronal and endocrine systems that interface with the immune system [1,2]. IL-1 is produced by various types of cells including macrophages, dendritic cells (DC), B cells and T cells [3]. It consists of two molecular species, IL-1α and IL-1β, which exert similar, although not completely overlapping, biological activities through the IL-1 type I receptor (IL-1RI; CD121a) [4]. Although an IL-1 type II receptor (IL-1RII; CD121b) has also been found, this receptor is not considered to be involved in the signal transduction, but considered to play more of a regulatory role as a "decoy" [4]. In addition, another member of the IL-1 gene family, the IL-1 receptor antagonist (IL-1Ra), binds to IL-1 receptors without exerting agonistic activity [4]. This molecule, together with IL-1RII and the secretory forms of IL-1RI and IL-1RII, are considered to be negative regulators of IL-1 signals, providing a complex regulation of IL-1 activity.

In the immune system, IL-1 is known to activate lymphocytes, monocytes, macrophages and NK cells [3,4]. When mice were immunized with protein antigens together with IL-1, serum Ab production was enhanced, suggesting that IL-1 has an adjuvant effect [5,6]. Recently, we found that IL-1Ra$^{-/-}$ mice developed chronic inflammatory arthropathy spontaneously and production of autoantibodies against Igs, type II collagen and dsDNA, increased in these mice [7]. These observations suggest an important role of IL-1 in the humoral immune responses. On the other hand, it was shown that humoral immune responses were normal in IL-1RI$^{-/-}$ mice [8,9]. Thus, the role of IL-1 in humoral immune response is still controversial.

In this report, I studied the roles of IL-1 in the humoral immune response using IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice. Ab production to SRBC was reduced in IL-1$^{-/-}$ mice, while it was enhanced in IL-1Ra$^{-/-}$ mice. I found that IL-1 was involved in T cell-priming because IL-1$^{-/-}$ APCs could not fully activated antigen-specific T cells. In addition, this response was independent on CD28-CD80/CD86 co-signaling. Furthermore, I showed that IL-1 produced by APCs enhances the expression of CD40L (CD154) and OX40 (CD134) on T cells, which play an important role in CD4$^+$ T cell-priming as well as antigen-specific B cells [11-15]. Since the defect of Ab production in IL-1$^{-/-}$ mice was rescued by the administration of agonistic anti-CD40 mAb, suggesting that IL-1 promotes humoral immune response by inducing these co-signaling molecules on T cells.

Materials and Methods

Mice

IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice were generated by homologous recombination as described previously and backcrossed to BALB/cA mice for 7 or 8 generations [10]. DO11.10 Tg mice (BALB/c background) were kindly provided by Dr. Dennis Y. Loh. All the 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 8 to 12 week-old adult mice were used for the experiments.

Immunization of mice

Mice were immunized with either 1×10^8 SRBC in PBS, intraperitoneally. Secondary responses were examined after immunization with SRBC. For *in vivo* reconstitution analysis, agonistic rat anti-mouse CD40 mAb (200 μg) (LB429) [16] or rat IgG (200 μg) was injected each time intraperitoneally at 1 day both after the primary and secondary immunization with SRBC. Goat anti-mouse IgM $F(ab')_2$ (200 μg) (ICN Biomedical, Inc, Aurora, OH) or goat IgM $F(ab')_2$ (200 μg) was administrated intraperitoneally at 1 day after the primary immunization with SRBC, then, rabbit anti-mouse IgG $F(ab')_2$ (200 μg) (Rockland, Gilbertsville, PA) or rabbit IgG $F(ab')_2$ (200 μg) was injected intraperitoneally at 1 day after the secondary immunization. Blood samples were collected from the tail vein before the immunization. At 15 days after the primary immunization, mice were given the secondary immunization and blood samples were collected 2 weeks later.

Measurement of Ab titers

Immunoglobulin levels in sera or culture supernatants were measured by ELISA as described previously [17]. Soluble SRBC antigen (2 μg/ml) which was prepared as described previously [18], was coated on Falcon 3912 Micro Test III™ Flexible Assay Plates (Becton Dickinson, Franklin Lakes, NJ). To measure OVA-specific Ab levels in culture supernatants or serum, 96-well plates for ELISA were coated with OVA peptide (10 μg/ml), and 50 μl of the test sample was added to each well. After incubation 1 h the well was washed with TBS+0.05% Tween 20 for 3 times, followed by the addition of 50 μl of alkaline phosphatase (AP)-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3 (ZYMED, San Francisco, CA), or AP-rat anti-mouse IgE (Southern Biotechnology

Associates, Inc., Birmingham AL). Alkaline phosphatase activity was measured using Substrate Phosphatase SIGMA104® (SIGMA, St. Louis, MO) as the substrate, and the absorbancy at 415 nm is shown.

Preparation of cells from lymphoid tissues

Cells were prepared from the spleen or lymph nodes (axillary, inguinal and brachial) by grinding the tissues with the plunger of a 1 ml disposable syringe, and were then suspended in a RPMI1640 (GIBCO BRL, Gaithersburg, MD) medium containing 50 μM 2-mercaptoethanol (GIBCO BRL), 50 μg/ml streptomycin (Meiji, Tokyo, Japan), 50 μg/ml penicillin (Meiji) and 10% fetal calf serum (FCS) (JRH BIOSCIENCE, Lenexa, KS). Spleen cells were treated with a hemolysis buffer (17 mM Tris.HCl, 140 mM NH_4Cl, pH 7.2) to remove red blood cells. Adherent cells and non-adherent cells were separated after incubation for 1 h on a 10 cm dish. For APCs in the primary T cell response assay, $B220^+$ and $Thy1.2^+$ cells were removed from splenic adherent cells (SACs) using a MACS column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To prepare splenic and lymph node T cells, non-adherent cells were passed through a nylon wool column. $CD4^+$ T cells were purified by treating the T cell preparation with anti-mouse CD8, anti-mouse B220, and anti-mouse Mac-1 magnetic beads (Miltenyi Biotec GmbH) and then passing them through a MACS column. B cells were prepared by treating splenic non-adherent cells with anti-Thy1.2 Ab (Serotec Ltd., Oxford, England) and rabbit complement (Cedarlane, Ontario, Canada). The purity of $CD4^+$ cells and $B220^+$ cells was monitored by FACScan and was usually about 90%.

T cell proliferative response

In the OVA-specific T cell proliferative response assay, splenic and lymph node $CD4^+$ T cells (5×10^4 cells/well) from DO11.10 Tg mice were co-cultured with irradiated-APCs (5×10^3 cells/well) for 3 days in the absence as well as in the presence of $OVA_{323-339}$ peptide (0.1 μM; gifted by Dr. Takashi Saito) in a final volume of 200 μl RPMI1640/10% FCS. The effects of recombinant mouse IL-1α (125 pg/ml) and IL-1β (125 pg/ml) (PEPROTECH, London, England), or CTLA-4 Ig (30 μg/ml) (gifted by Dr. Ryo Abe) were examined by incubating the culture with those Abs for 72h, 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 [^{3}H]-thymidine radioactivity in the acid-insoluble fraction was measured with Micro Beta™(Pharmacia Biotech, Piscataway, NJ).

B cell proliferative response

Splenic B cells (1×10^5 cells/well) and mitomycin C (SIGMA)-treated DO11.10 T cells (1×10^5 cells/well) were cocultured for 3 days in the presence or absence of the OVA peptide (0.2 μM), and cells were labeled with [^{3}H] thymidine for 6 h. In order to examine the effects of agonistic anti-mouse CD40 mAb (HM40-3; 1 μg/ml) (PharMingen), cells were cultured for 3 days with this antibody and proliferation and OVA-specific Ab levels were measured. Isotype IgG was used as a control.

Measurement of cytokine levels

IL-2 levels in the culture supernatant were determined by Titer Zyme EIA kit (PerSeptive Diagnostics, Inc., Cambridge, MA). As a standard recombinant cytokine, mouse IL-2 (Genzyme, Cambridge, MA) was used. TMB One-Step Substrate System was purchased from DAKO (Carpinteria, CA).

Flow cytometric analysis

In the OVA-specific T cell and B cell proliferative response, cells were harvested at the time-point when expression of each molecule reached peak levels. Staining of I-A^d (at 72 h after stimulation), CD80 (72 h), CD86 (72 h) and CD40 (72 h) on SACs, CD40L (12 h), OX40 (72 h) and IL-2Rα (60 h) on $CD4^+$ T cells and OX40L (72 h) on B cells were performed according to the standard protocol. Detection of CD40L on KJI-26^+CD4^+T cells was carried out as described previously [19]. Briefly, the biotin-labeled anti-mouse CD40L mAb was added to OVA-specific T cell proliferation culture, and at 12 h after stimulation, cells were harvested and stained with PE-anti-mouse CD4 mAb, anti-mouse DO11.10 (KJI-26) and CyChrome-streptavidin (PharMingen). After washing, cells were stained with second antibody, FITC-anti-mouse Ig (PharMingen). In order to examine the effects of rIL-1 on CD40L and OX40 expression on $CD4^+$ T cells, $CD4^+$ T cells (1×10^6 cells/well) were cultured with either rIL-1 only, or with plate-coated anti-CD3 (145-2C11) (0.1 μg/ml) in the presence or absence of rIL-1, or with plate-coated anti-CD3 (0.1 μg/ml) plus soluble anti-mouse CD28 (PV-1, a gift from Dr. Abe) (1 μg/ml). To detect IL-1RI expression on $CD4^+$ T cells, $CD4^+$ T cells (1×10^6 cells/well) were cultured with plate-coated anti-CD3 (0.1 μg/ml) in the presence or absence of soluble anti-mouse CD28 (1 μg/ml). Cells were incubated for 12 h for the analysis of CD40L and IL-1RI expression and 72 h for OX40 expression.

Anti-mouse CD16/CD32 (2.4G2), FITC or PE-anti-mouse CD4 (GK1.5), PE-anti-

mouse B220 (RA3-6B2), PE-anti-mouse CD25 (IL-2Rα) (3C7), biotinylated anti-mouse I-A^d (AMS-32.1), biotinylated anti-mouse CD121a (IL-1RI)(12A6) and FITC-streptavidin were purchased from PharMingen, and FITC-anti-mouse CD80 (16-10A1) was from BioSource (Camarillo, CA). PE-anti-mouse CD40 (3.23) and PE-anti-mouse OX40 (OX86) were from Immunotech (Marseille Cerdex, France). Anti-mouse CD86 (GL-1) mAb was kindly provided by Dr. Hideo Nariuchi. Biotinylated anti-mouse OX40L, MGP34, was kindly provided by Dr. Kazuo Sugamura and RM134L was purchased from PharMingen.

Statistics

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

Results

Ab production to SRBC in IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice

Although adjuvant effects of IL-1 are well known on Ab production, it is not clear if IL-1-deficiency causes any defects in humoral immune response because Ab production was normal in IL-1RI$^{-/-}$ mice immunized TNP-KLH together with alum or complete Freund's adjuvant (CFA). Thus, I examined whether or not IL-1 is involved in Ab production using IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice of BALB/c background. After immunization with SRBC intraperitoneally, SRBC-specific serum Ab levels were measured by enzyme-linked immunosorbent assay (ELISA). SRBC-specific Ab levels of IgM, IgG and IgE classes in IL-1$^{-/-}$ mice were significantly lower than those in wild-type mice after secondary immunization (Fig. 1A). In contrast, SRBC-specific IgG and IgE levels in IL-1Ra$^{-/-}$ mice were increased compared with wild-type mice, although IgM levels were comparable in both mice (Fig. 1B). The suppression in IL-1$^{-/-}$ mice and the augmentation in IL-1Ra$^{-/-}$ mice were observed in all the IgG subclasses, showing no polarization to either Th1 or Th2 type response.

The physiological levels of serum immunoglobulins (IgM, IgG and IgE) without immunization were similarly low in these IL-1$^{-/-}$ mice (data not shown). These results indicate that IL-1 plays an important role in T cell-dependent Ab production under physiological conditions.

I did not detect any difference in the number and composition of the immune cells from the thymus, spleen, lymph nodes and peritoneal cavity between IL-1$\alpha/\beta^{-/-}$ and wild-type mice, when I examined various cell surface markers (CD4 and CD8 on thymocytes; CD4, CD8, CD3ε, B220, CD62L and CD44 on lymph node cells; CD4, CD8, CD3ε, B220, IgM, CD11b, CD11c, CD80, CD86, I-A^d, CD54, CD40, CD16/CD32, CD21/CD35, CD62L and CD44 on splenocytes; B220, IgM, CD11b, F4/80, CD16/CD32, CD21/CD35 and CD5 on peritoneal cells) (data not shown). This indicates that IL-1 does not affect the development and maturation of T cells, B cells, and APCs. Intrinsic B cell functions such as proliferative response to LPS or anti-IgM mAb, and Ab production against T-independent antigen TNP-LPS, was normal in IL-1$^{-/-}$ mice (data not shown). Intrinsic T cell functions such as proliferative response and cytokine production to plate-coated anti-CD3 mAb or plate-coated anti-CD3 mAb plus soluble anti-CD28 mAb were also normal in IL-1$^{-/-}$ mice (data not shown). Moreover, phagocytic activity of Mϕs and DCs of IL-1$^{-/-}$ mice was comparable with that of wild-type mice using FITC-latex beads, FITC-dextran and lucifer yellow. Antigen processing ability of these cells was also normal (data not shown). These

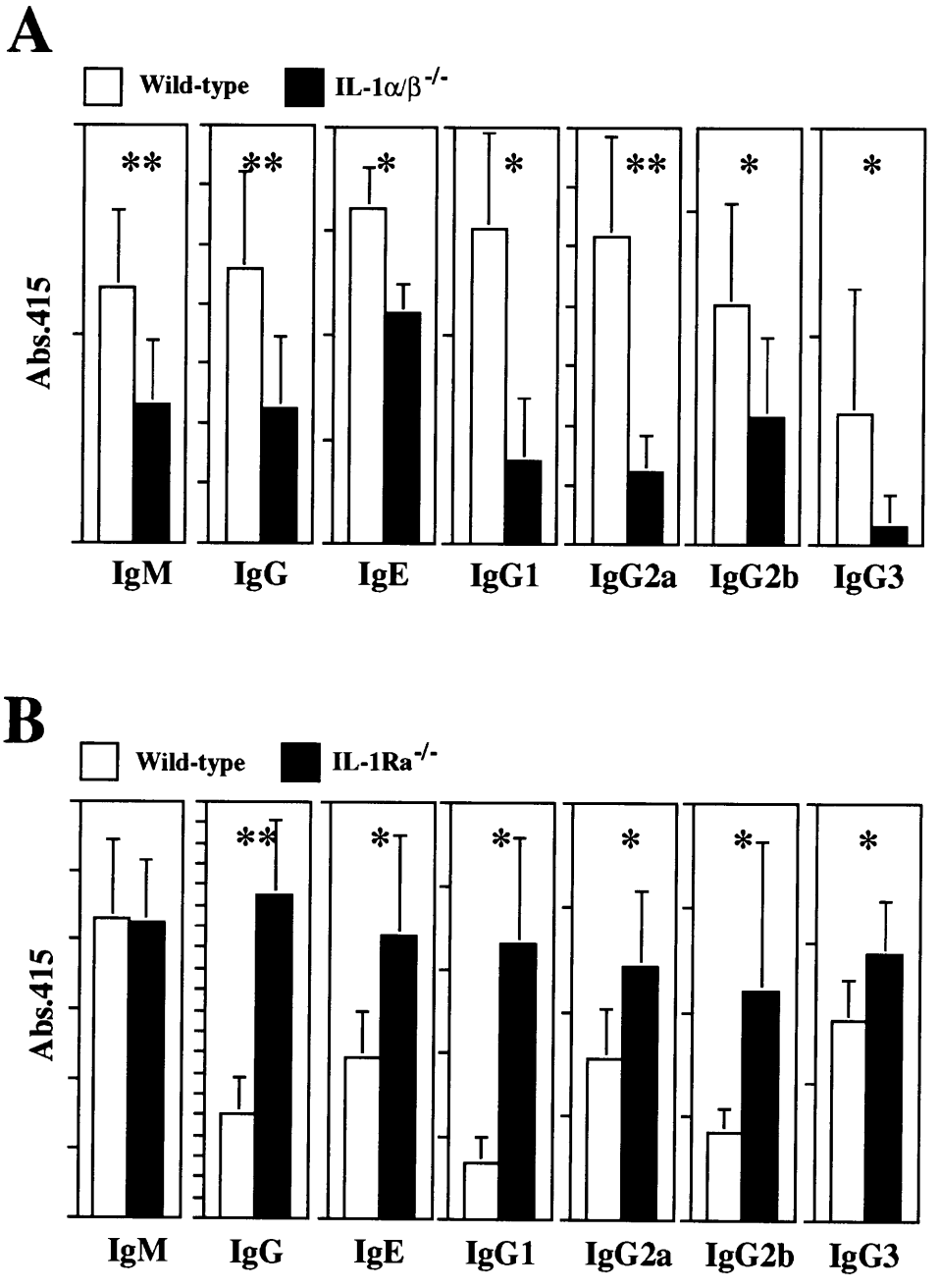

Figure 1. Efficiency of Ab production against SRBC in IL-1-/- and IL-1Ra-/- mice
Mice were immunized with SRBC, and sera were collected 2 weeks after the secondary immunization. After appropriate dilution of the serum (IgM, IgG: 1/100; IgE: 1/2; IgG1: 1/100; IgG2a, 2b, 3: 1/10), SRBC-specific Ab levels in the sera were measured by ELISA. (A) wild-type mice: n=10, IL-1-/- mice: n=10. (B) IL-1Ra-/- mice: n=7. The average and standard deviation (SD) are shown. A graduation of the ordinate (Abs.415) is 0.1. * $p<0.05$, and ** $p<0.005$.

results indicate that intrinsic B cell, T cell and APC function was not affected by the deficiency of IL-1.

The role of IL-1 in T cell-APC interaction

Then, I examined roles of IL-1 in T cell-APC interaction. In order to assess roles of IL-1 in T cell-priming upon interaction with APCs, the antigen-specific primary T cell proliferative response was assayed using T cells from DO11.10 transgenic (Tg) mice, who express TCR specific for the OVA323-339 peptide, and splenic adherent cells (SACs) from IL-$1^{-/-}$ mice. The proliferative response of DO11.10 T cells was reduced in IL-$1^{-/-}$ SACs (Fig. 2A). On the other hand, using IL-$1Ra^{-/-}$ SACs, the response was slightly increased (Fig. 2A) (wild-type: 100% vs. IL-$1^{-/-}$: 41% ± 10%, $p < 0.01$; IL-$1Ra^{-/-}$: 135% ± 12%, $p < 0.05$. Average ± SD from three independent experiments). When recombinant mouse IL-1α and IL-1β (rIL-1) were added to this culture, the response with IL-$1^{-/-}$ SACs was recovered, indicating that the defect is not developmental (Fig. 2B). Similar IL-1-dependent activation of T cells was observed when B cells were used as APCs (data not shown). These results suggest that IL-1 from SACs play an important role in T cell-priming.

Effects of IL-1-deficiency on the expression of cell surface molecules on lymphocytes

Because the above mentioned results have suggested that T cell activation through T cell-APC interaction is impaired in IL-$1^{-/-}$ mice, I examined the molecules involved in cell-cell interaction on APCs and T cells. The expression levels of I-A^{d} on SACs from IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice were comparable to those from wild-type mice (Fig. 3A). The expression levels of CD80 and CD86 on I-A^{d+} APCs were also similar among these mice (Fig. 3A). Moreover, in spite of the inhibitory effect of CTLA-4 Ig, which inhibits CD28-CD80/CD86 co-signaling, T cell responses were still reduced with IL-$1^{-/-}$ APCs and enhanced with IL-$1Ra^{-/-}$ APCs (Fig. 3B) (wild-type: 100% vs. IL-$1^{-/-}$: 29% ± 10%, $p < 0.001$; IL-$1Ra^{-/-}$: 199% ± 29%, $p < 0.05$. Average ± SD from three independent experiments). In these cultures, IL-2 levels well correlated with the proliferative response, consistent with the impairment of T cell activation (Fig. 3B). These results indicate that IL-1 acts on T cell-priming independent on CD28-CD80/CD86 co-stimulatory signals.

I next investigated expression of CD40-CD40L and OX40-OX40L, which are also suggested to be involved in T cell-priming. The expression levels of CD40 on I-A^{d+} APCs were normal both in IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice (Fig. 3A). On the other hand, the

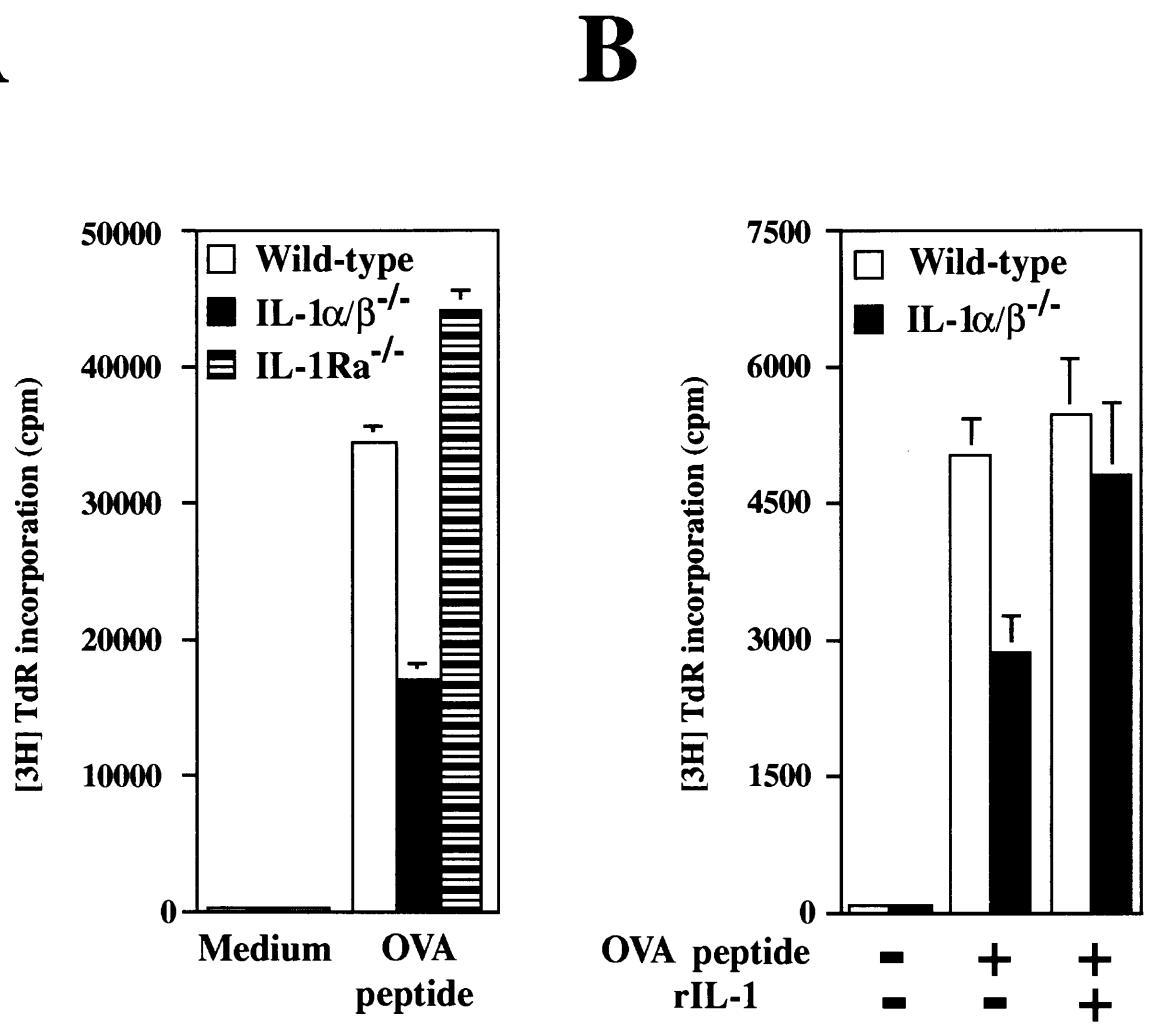

Figure 2. Effects of IL-1 on primary T cell proliferative response
Proliferative responses against the OVA323-339 peptide were assessed by measuring the incorporation of [3H] thymidine after 3 days culture. (A) Effects of IL-1 on T cell-priming were evaluated using DO11.10 T cells and SACs from wild-type, IL-1-/- and IL-1Ra-/- mice. (B) Effects of exogenous IL-1 (rIL-1α/β) on T cell-priming were examined using DO11.10 T cells and SACs from wild-type and IL-1-/- mice. Average ± SD of triplicate experiments is shown. These results were reproducible three independent experiments.

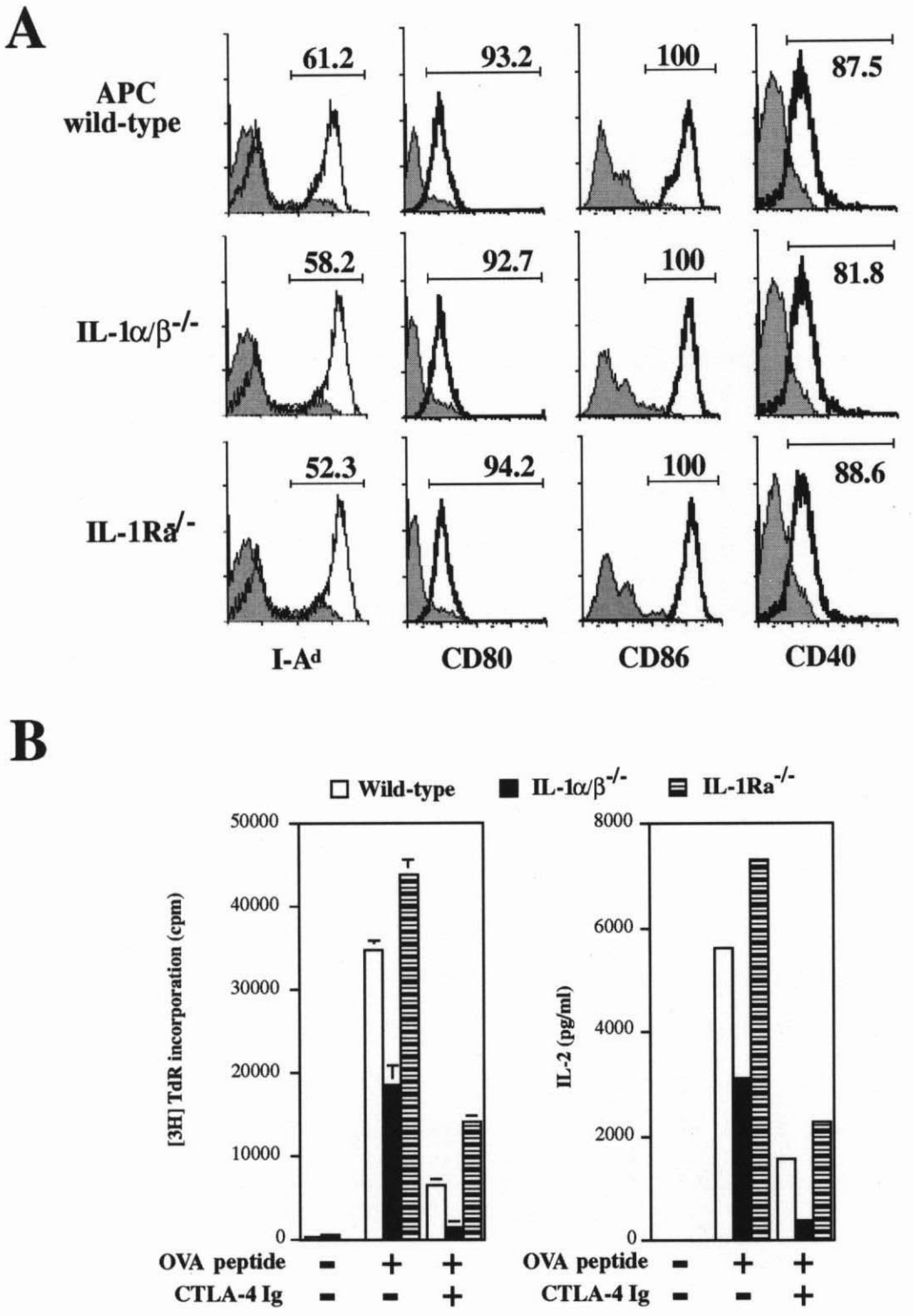

Figure 3. Co-stimulatory activity of IL-1 independent on CD28-CD80/CD86 pathway
(A) Expression levels of I-Ad on B220-negative SACs from wild-type, IL-1-/- and IL-1Ra-/- mice, and CD80, CD86 and CD40 on I-Ad+ SACs from wild-type, IL-1-/- and IL-1Ra-/- mice were analyzed by flow cytometry under the conditions of primary T cell proliferation assay described in Figure 2a. Shaded area shows isotype matched control Ig stainings. (B) Effects of CTLA-4 Ig on primary T cell response were assessed using DO11.10 T cells and SACs from wild-type, IL-1-/- and IL-1Ra-/- mice. Average ± SD of triplicate experiments is shown. IL-2 levels in pooled triplicate supernatant from proliferative response assay were examined by ELISA. These results were reproducible three independent experiments

expression levels of CD40L and OX40 on $CD4^+$ DO11.10 T cells stimulated with IL-$1^{-/-}$ APCs were low compared with wild-type APCs (Fig. 4A and 4B) (CD40L, wild-type: 100% vs. IL-$1^{-/-}$: 68% ± 2%, $p < 0.01$; OX40, 56% ± 16%, $p < 0.01$. Average ± SD from three independent experiments). In contrast, the expression levels of these molecules on T cells were enhanced when IL-$1Ra^{-/}$ APCs were used (Fig. 4B) (CD40L, wild-type: 100% vs. IL-$1Ra^{-/-}$: 154% ± 10%, $p < 0.01$; OX40, 133% ± 7%, $p < 0.01$. Average ± SD from three independent experiments). In support for the involvement of IL-1 in the CD40L induction, I found that this reduced CD40L expression could be rescued by the addition of recombinant mouse IL-1α and IL-1β in the culture (Fig. 5A). In addition, the expression level of IL-2Rα (CD25), an activation marker of T cells, on $CD4^+$ DO11.10 T cells upon incubation with IL-$1^{-/-}$ APCs was reduced, and that with IL-$1Ra^{-/-}$ APCs was enhanced compared with wild-type APCs (Fig. 5A) (wild type: 100% vs. IL-$1^{-/-}$: 71% ± 4%, $p < 0.01$; IL-$1Ra^{-/-}$: 123% ± 10%, $p < 0.005$. Average ± SD from three independent experiments) (Fig. 4A and 4B). The expression levels of IL-1RI on $CD4^+$ DO11.10 T cells cultured with IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ APCs did not differ from that observed with wild-type APCs (data not shown). These observations suggest that IL-1 produced by APCs plays a crucial role in T cell-priming by enhancing the expression of CD40L and OX40.

Then, I examined whether or not IL-1 directly induces CD40L and OX40 expression on $CD4^+$ T cells. I found that rIL-1 did not induce CD40L and OX40 expression on naive $CD4^+$ T cells. However, activation of T cells with plate-coated anti-CD3 mAb (0.1 μg/ml) made these cells responsive to rIL-1 in a dose dependent manner (Fig. 5B). This effect of rIL-1 was not observed when high concentrations of anti-CD3 mAb (1 and 10 μg/ml) were used (data not shown). High doses of rIL-1 (10 and 100 ng/ml) were rather inhibitory on the induction of CD40L and OX40 expression (data not shown). I found that IL-1RI was not expressed on naive $CD4^+$ T cells, and it was induced by the treatment with anti-CD3 mAb (Fig. 5C). Thus, these results clearly show that IL-1 directly induces CD40L and OX40 expression on $CD4^+$ T cells, although induction of IL-1RI through TCR signaling is necessary in advance.

Dependency on CD40-CD40L signaling in IL-1-deficient mice

I, then, examined whether or not activation of CD40 can recover the antigen-specific B cell response in IL-1-deficient mice. When splenic B cells from wild-type and IL-$1^{-/-}$ mice were cultured with mitomycin C-treated T cells from DO11.10 Tg mice in the presence of the

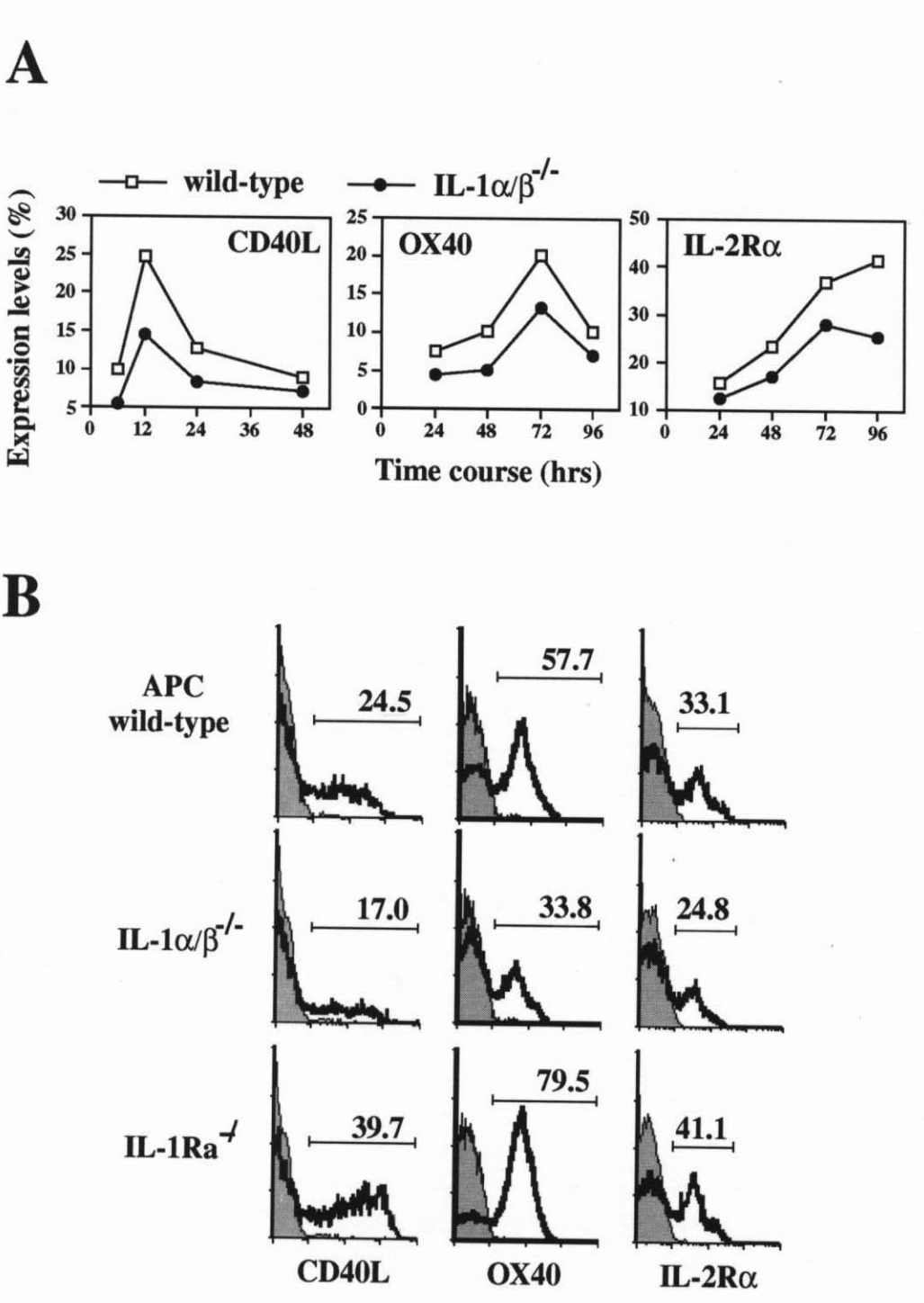

Figure 4. Effects of IL-1 deficiency on the expression of cell surface molecules on T cells
The expression levels of surface molecules on CD4+ DO11.10 T cells after stimulation with $OVA_{323-339}$ peptide in the presence of either wild-type, IL-1-/- or IL-1Ra-/- APCs were analyzed by flow cytometry. (A) The time kinetics of CD40L, OX40 and IL-2Ra induction on CD4+ DO11.10 T cells. Open squares were wild-type APCs and closed circles were IL-1-/- APCs. The results were confirmed in another experiment. (B) Expression levels of CD40L, OX40, and IL-2Ra on CD4+ DO11.10 T cells at the peak time points. The expression of CD40L, OX40, and IL-2Ra was analyzed by flow cytometry after 12 h, 60h, and 72 h, respectively. Shaded area shows isotype matched control Ig stainings. One of three representative results is shown.

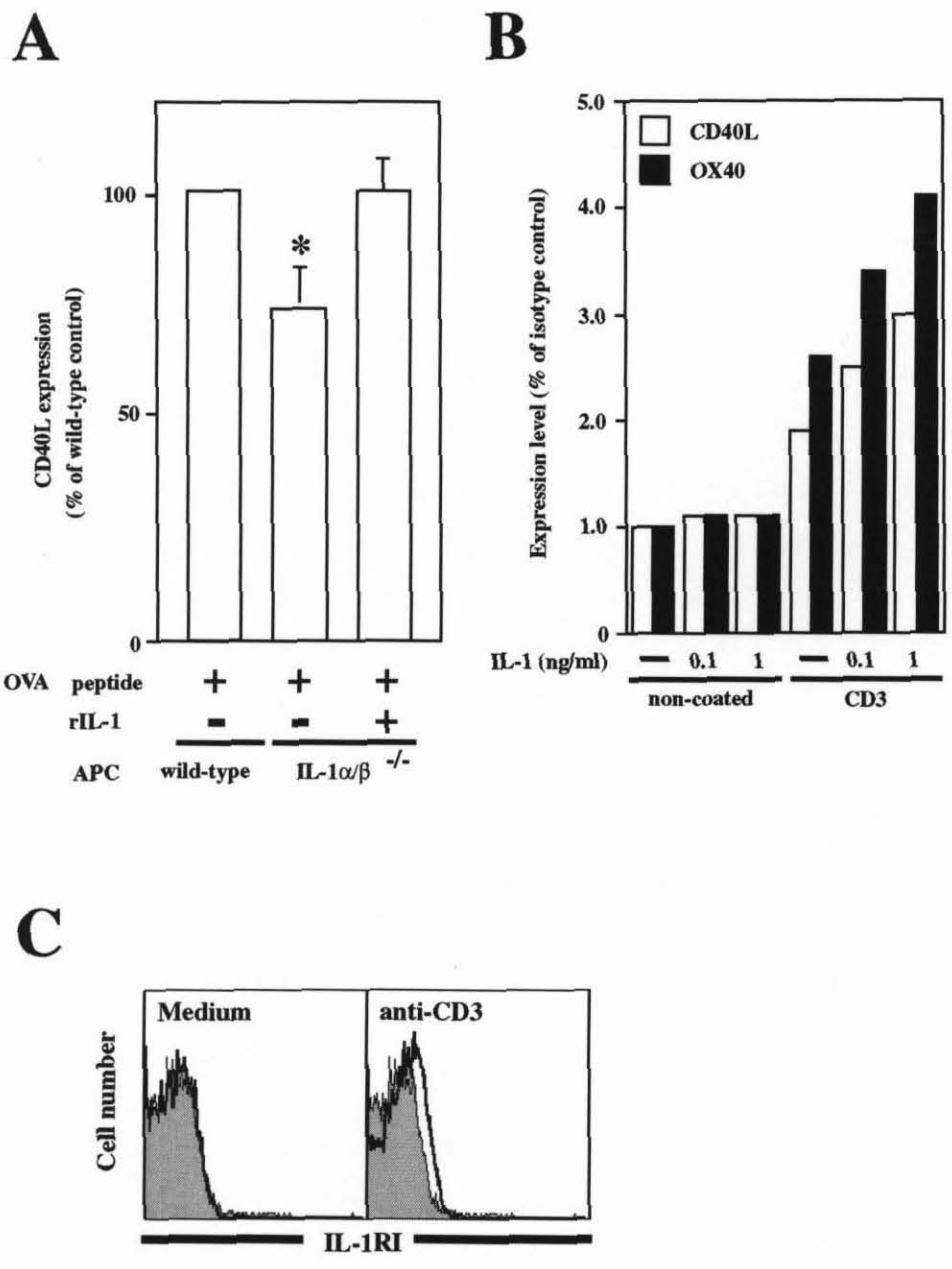

Figure 5. Induction of CD40L and OX40 expression on CD4+ T cells with rIL-1
(A) The defect of CD40L expression on CD4+ DO11.10 T cells cultured with IL-1α/β-/- APC is rescued by rIL-1α/β treatment. A relative value of CD40L-positive T cells in IL-1α/β-/- APC culture against those in wild-type APC culture is shown. An average SD± of four independent experiments is shown. * $p < 0.01$. (B) Naïve T cells are not responsive to rIL-1 treatment. Purified splenic CD4+ T cells were treated with rIL-1 with or without stimulation with plate-coated anti-CD3 mAb (0.1 μg/ml). Expression of CD40L and OX40 on CD4+ T cells was analyzed by flow cytometry at 24 h and 72 h after the treatment, respectively. Expression levels of CD40L and OX40-positive T cells relative to the isotype-matched control Ig staining are shown. (C) IL-1RI is induced on T cells by the treatment with plate-coated anti-CD3 mAb. Purified splenic CD4+ T cells were stimulated with plate-coated anti-CD3 mAb (0.1 μg/ml). After 24 h, expression of IL-1RI on CD4+ T cells was examined by flow cytometry. Shaded area shows isotype-matched control Ig staining.

OVA peptide, the proliferative response of that from IL-$1^{-/-}$ mice was reduced compared with wild-type mice (Fig. 6A, in the presence of control IgG) (wild-type: 100% vs. IL-$1^{-/-}$: 48% ± 12%, $p < 0.05$. Average ± SD from three independent experiments). The OVA-specific Ig levels in the culture supernatant of IL-$1^{-/-}$ B cells were also reduced to 40% (Fig. 6A). Under this culture condition, the CD40L-expressing T cell population was less in the culture with IL-$1^{-/-}$ B cells than in the culture with wild-type B cells (Fig. 6B). Moreover, OX40L expression on IL-$1^{-/-}$ B cells was reduced compared to that of wild-type B cells, indicating that IL-$1^{-/-}$ B cells were activated only weakly (wild-type: 100% vs. IL-$1^{-/-}$: 69% ± 3%, $p < 0.01$. Average ± SD from three independent experiments) (Fig. 6C). Since CD40 activation is necessary for the induction of OX40L on B cells [20], this result is consistent with the observation that CD40L expression was reduced on T cells activated with IL-1-deficient APCs. Then, I tried to recover the immune response of IL-$1^{-/-}$ B cells by treating cells with agonistic anti-CD40 mAb. As shown in Fig. 6D, the reduced proliferative response and Ab production of the mutant B cells were recovered to the normal levels when agonistic anti-CD40 mAb was added to the culture.

The enhancing effect of anti-CD40 mAb was also observed *in vivo*; SRBC-specific Ab production was recovered to the wild-type levels, when anti-CD40 mAb was administered to IL-$1^{-/-}$ mice during SRBC immunization (Fig. 7A). On the other hand, anti-IgM F(ab')2 plus anti-IgG F(ab')2 administration was not effective to recover SRBC-specific IgG production in IL-$1^{-/-}$ mice to the wild-type levels, although SRBC-specific IgG levels were increased both in IL-$1^{-/-}$ and wild-type mice (Fig. 7B). Thus, it was shown that IL-1 can be substituted by CD40 activation. These results suggest that IL-1 produced by APCs plays an important role in T cell-priming and Ab production by enhancing the expression of CD40L and OX40 on T cells.

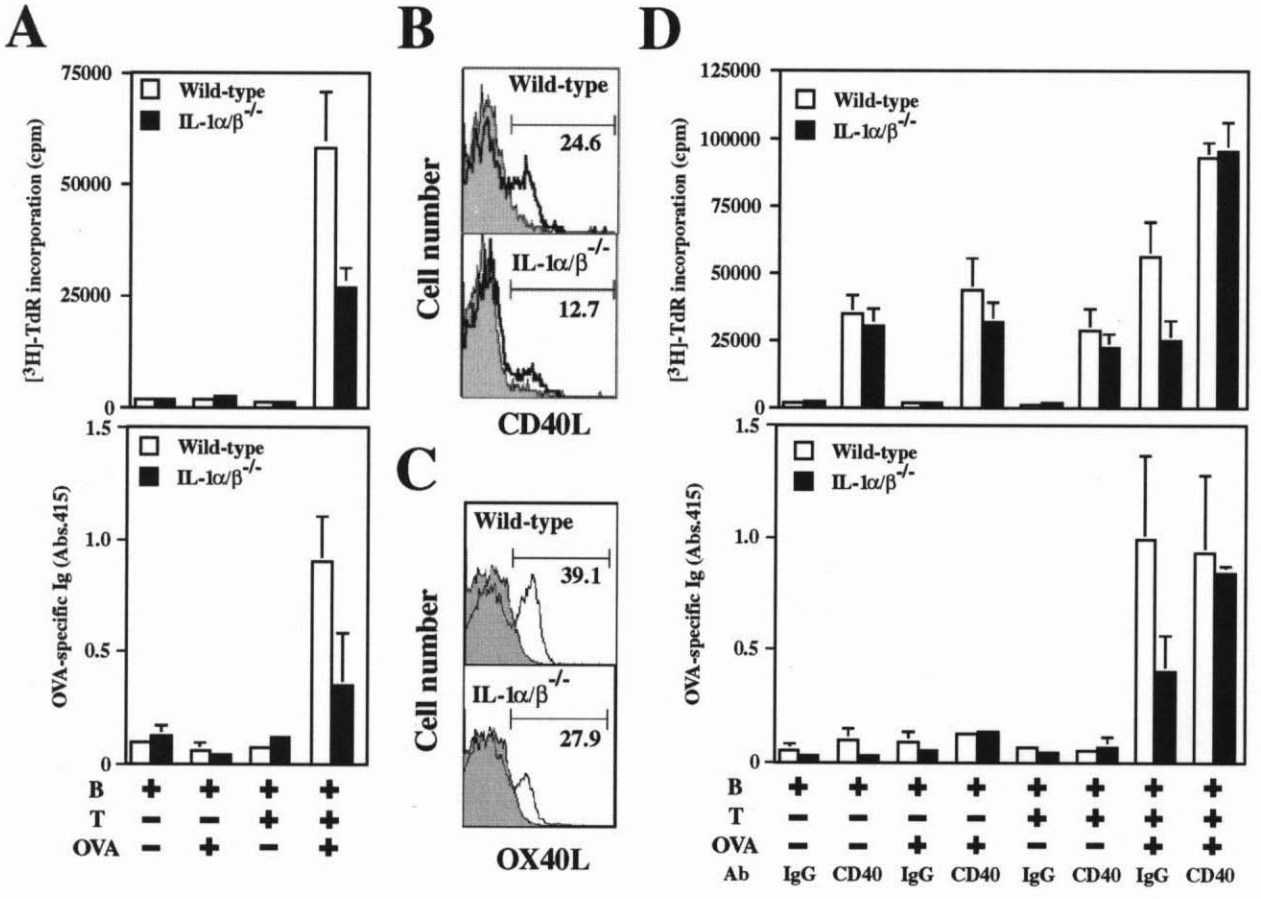

Figure 6. Rescue of the immune response of IL-1-/- B cells by agonistic anti-CD40 mAb treatment

(A) OVA-specific immune response is impaired in IL-1-/- B cells and is rescued by agonistic anti-CD40 mAb teatment. Splenic B cells from wild-type and IL-1-/- mice were cultured with mitomycin C-treated CD4+ DO11.10 T cells in the presence or absence of OVA peptide. Then, effects of agonistic anti-CD40 mAb or control isotype IgG on B cell proliferation (upper panel) and OVA-specific antibody production (lower panel) were measured. An average of triplicate and SD are shown. The result was confirmed in another experiment. (B) CD40L expression levels in T cells cultured with IL-1-/- B cells. CD4+ DO11.10 T cells were cultured as described in (A), then, CD40L expression was analyzed by flow cytometry after 24 h. Shaded area represents isotype-matched control Ig staining. One of three representative resutls is shown. (C) OX40L expression levels in IL-1-/- B cells. B cells were cultured as described in (A), and after 72 h, OX40L expression was analyzed by flow cytometry. Shaded area shows isotype-matched control Ig staining. One of representative data from three-independent experiments is shown.

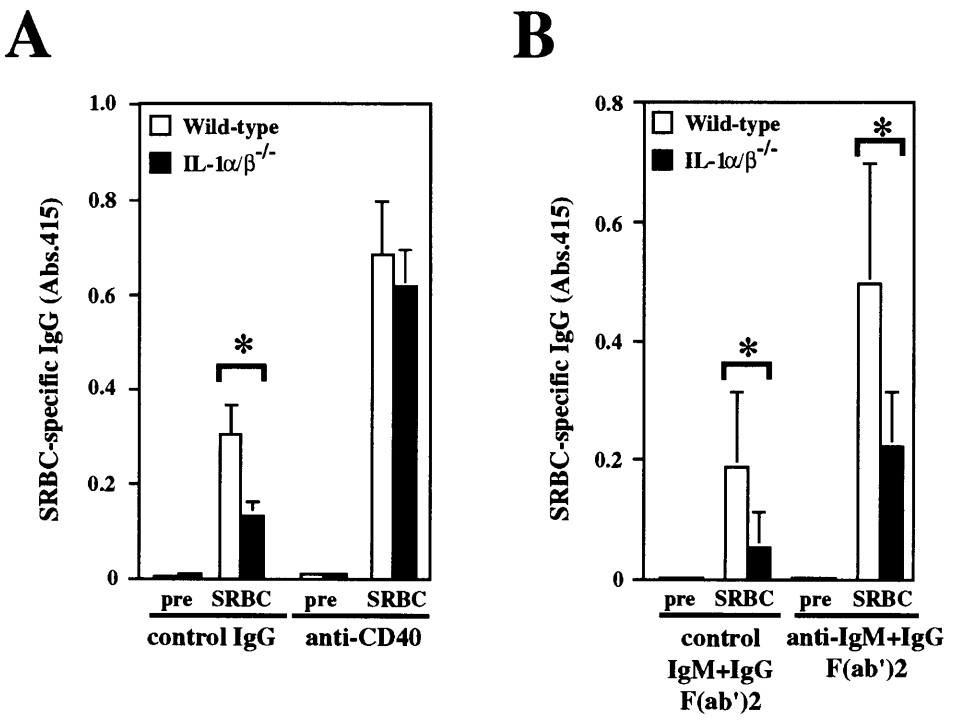

Figure 7. Recovery of SRBC-specific Ab production in IL-1-/- mice by agonistic anti-CD40 mAb treatment

Rescue of Ab production against SRBC in IL-1-/- mice by the injection with anti-CD40 mAb (A) or anti-IgM F(ab')2 plus anti-IgG F(ab')2 (B). Mice were immunized with SRBC, then injected with anti-CD40 mAb or isotype IgG 24 h after the primary and secondary immunization. Sera were collected before immunization (pre) and 1 week (SRBC) after the secondary immunization. SRBC-specific IgG levels in the sera were measured by ELISA. An average and SD are shown. (A) Wild-type: n=6, IL-1-/-: n=7 (B) Wild-type: n=8, IL-1-/-: n=8 * $p<0.05$

Discussion

In this report, I analyzed the mechanisms of humoral immune response activation by IL-1 using IL-$1^{-/-}$ and IL-$1Ra^{-/-}$ mice, and have shown that IL-1 plays an important role in enhancing T cell-APC interaction through inducing CD40L and OX40 on T cells. The intrinsic functions of B cells, T cells, and APCs from IL-$1^{-/-}$ mice were normal. However, the proliferative response as well as IL-2 production of $CD4^+$ DO11.10 T cells against OVA peptide was reduced when IL-$1^{-/-}$ SACs were used as APCs, suggesting that T cell-APC interaction was impaired in IL-$1^{-/-}$ mice. I found that the expression levels of CD40L and OX40 were reduced in the co-culture of $CD4^+$ DO11.10 T cells with IL-$1^{-/-}$ APCs. Furthermore, I showed that rIL-1 added exogenously to the culture can induce the expression of CD40L on $CD4^+$ T cells, indicating that IL-1 is an inducer of this co-signaling molecules. Since agonistic anti-CD40 mAb could rescue the deficiency observed in IL-$1^{-/-}$ mice both *in vivo* and *in vitro*, I conclude that IL-1 enhances T cell-priming through induction of co-signaling molecules that are important in both T cell-APC and T cell-B cell interactions.

The importance of CD40-CD40L interaction in T cell-priming and B cell activation has been amply documented [11-13] [21]. It has also been shown that neither $CD40^{-/-}$ mice nor $CD40L^{-/-}$ mice are able to react with TD antigens to produce IgG Ab efficiently [11,22,23]. $CD40L^{-/-}$ mice also showed a profound reduction in the primary IgM antibody responses to SRBC [22], although the IgM anti-KLH response was not completely absent in $CD40L^{-/-}$ mice.

The OX40-OX40L signaling system has also been suggested to play an important role in the humoral immune response. OX40 ligation with OX40L activates naive T cells to produce Th2 cytokines and differentiate into Th2 cells [24] [25], and promotes Ab production against TD antigens [26]. Murata et al. (2000) reported that antibody production against KLH was impaired in $OX40L^{-/-}$ mice. However, other investigators reported that serum antigen-specific Ig levels were similar to wild-type mice when $OX40^{-/-}$ or $OX40L^{-/-}$ mice were immunized with various TD antigens including VSV, LCMV, TMEV, *L. major*, *N. brasiliensis*, NP-CGG, TNP-KLH and TNP-OVA, indicating that the OX40-OX40L system is not required under certain conditions of immunization [27-29]. These observations indicate that the CD40L-CD40 and OX40-OX40L co-signaling systems play an important, but not absolute, role in T cell-priming and Ab production. Thus, it is suggested that the inefficiency of T-dependent Ab production and T cell-priming in IL-$1^{-/-}$ mice is caused by the reduced expression of CD40L and OX40 on T cells upon interaction with IL-$1^{-/-}$ APCs. In

support for this notion, I showed that the defects in T cell-APC interaction could be rescued by the addition of agonistic anti-CD40 Ab both *in vivo* and *in vitro*.

The CD28-CD80/CD86 co-signaling system is known to be important for T cell proliferation and cytokine secretion in humoral immune responses [30]. Both primary and secondary T cell responses, and Th2 type cytokine secretion are impaired in CD80/CD86$^{-/-}$ mice [31]. However, the expression levels of CD80 and CD86 on APCs were normal in IL-1$^{-/-}$ mice. Moreover, I showed that CTLA-4 Ig suppressed CD4^{+} DO11.10 T cell proliferation independently of the IL-1-deficiency. These results strongly suggest that IL-1 has a previously unknown T cell activation mechanism differs from CD28-CD80/CD86 system.

IL-1 function in the humoral immune response has been recently examined using IL-1RI$^{-/-}$ mice [8,9]. These reports showed that specific serum Ab levels were normal in IL-1RI$^{-/-}$ mice when these mice were immunized with TNP-KLH/alum or TNP-KLH/CFA either intraperitoneally or subcutaneously. Furthermore, they showed that KLH-specific secondary T cell proliferative responses were normal in these mice. These results apparently contradict our findings. This discrepancy is not likely due to a difference in the mouse strains used in the experiments, because I obtained the same results using IL-1$^{-/-}$ mice on the C57BL/6 background as that obtained on the BALB/c background mice. Another possibility could be that there were differences in the immunization method.

Many studies indicate that antigen dosage, adjuvant, and the route of immunization (e.g. intraperitoneal, intravenous, or subcutaneous) affect the efficiency of the immune response and the Ig subclasses of the Abs. Different immune responses depending on the immunization program employed among investigators have been reported using CD80/CD86$^{-/-}$ mice [32], IL-6$^{-/-}$ mice [33-36], TNF$\alpha^{-/-}$ mice [17], and OX40$^{-/-}$ and OX40L$^{-/-}$ mice as it was described previously [20,27-29]. Regarding this, it has been reported that the subcutaneous route of immunization may cause local inflammation [37,38]. Adjuvants also cause inflammation at the site of injection, which could potentially induce the production of various inflammatory cytokines including IL-1. Since the functions of these inflammatory cytokines overlap partially, it is conceivable that the adjuvant effect of IL-1 could be substituted by some other cytokines such as TNF-α or IL-6. In a recent study, it was reported that using alum as an adjuvant could induce Th2 responses independently from IL-4- and IL-13-mediated signals [39]. It is conceivable that the effects of IL-1-deficiency might not be observed when mice were immunized with adjuvant or soluble antigens, as in the reports by Glaccum et al. (1997) and Satoskar et al. (1998).

Another possibility is that the discrepancy is caused by a difference between protein

antigens and particle antigens. With regard to this, it was reported that SRBC-specific IgG production was impaired in LTα$^{-/-}$ mice, which show deficiencies in LNs formation, splenic microarchitecture, germinal center formation, and follicular dendritic cell (FDC) network. Whereas IgG production against high dose NP-OVA was observed normally in this mutant mouse, suggesting that antibody production against SRBC depends on the FDC clustering while that against NP-OVA is not [40-42].

This discrepancy is not caused by the difference of the mutant mice I used. I examined SRBC-specific antibody production in IL-1RI$^{-/-}$ mice without using any adjuvant, and found that the immune response was reduced both in IL-1RI$^{-/-}$ and IL-1$^{-/-}$ mice of the C57BL/6J background and accelerated in IL-1Ra$^{-/-}$ mice of the same background (data not shown).

In conclusion, these observations indicate that IL-1 is a potent activator of the humoral immune response, and that IL-1Ra has important regulatory functions in the immune system. Immune modulating activity via IL-1 is clearly of benefit for host defenses, as IL-1 produced upon infection with bacteria or viruses would enhance the immune response against these pathogens. Any deficiency of the IL-1/IL-1Ra system, then, will likely cause serious problems in immunologic response. Our recent finding that IL-1Ra$^{-/-}$ mice develop autoimmune arthritis supports for this idea [7]. This suggests that the balance between IL-1 and IL-1Ra is of great importance in maintaining the homeostasis of the immune system. Involvement of IL-1/IL-1Ra in various autoimmune diseases, such as rheumatoid arthritis, ulcerative colitis, systemic lupus erythematosus, psoriasis, lichen sclerosus, alopecia areata, and Sjogren's syndrome has also been suggested [1,43]. Further elucidation of the control mechanisms of the IL-1/IL-1Ra system should provide us with important cues in the quest to develop therapeutics for these diseases.

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CHAPTER II

IL-1β, but not IL-1α, Is Required for T Cell-dependent Antibody Production

Summary

Interleukin-1 (IL-1) is consisted of two molecules, IL-1α and IL-1β, and IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of these molecules. Although adjuvant effects of exogenously administered IL-1 in humoral immune response is well known, roles of endogenous IL-1 and functional discrimination between IL-1α and IL-1β have not been elucidated completely. In this report, I investigated that the role of IL-1 in the humoral immune response using gene-targeted mice. Both primary and secondary Ab production against T-dependent antigen, sheep red blood cells (SRBC), was significantly reduced in IL-1α/β$^{-/-}$ mice, and enhanced in IL-1Ra$^{-/-}$ mice. The intrinsic functions of B cells such as Ab production against type1 T-independent antigen, trinitrophenyl (TNP)–LPS, and proliferative responses against mitogenic stimuli were normal in IL-1α/β$^{-/-}$ mice. Proliferative response of T cells and cytokine production upon stimulation with anti-CD3 mAb were also normal, as was the phagocytotic ability of antigen-presenting cells (APCs). However, SRBC-specific proliferative response and cytokine production of T cells through the interaction with APCs were markedly impaired in IL-1α/β$^{-/-}$ mice, and enhanced in IL-1Ra$^{-/-}$ mice. Moreover, I show that SRBC-specific Ab production was reduced in IL-1β$^{-/-}$ mice, but not in IL-1α$^{-/-}$ mice. These results show that endogenous IL-1β, but not IL-1α, is involved in T cell-dependent Ab production, and IL-1 promotes the antigen-specific T cell helper function through the T cell-APC interaction.

Introduction

Interleukin-1 (IL-1) has been found to be involved in various reactions including inflammation, acute phase responses, host defense against bacterial and viral infection, fever development, and stress responses [1,2]. IL-1 has two forms, IL-1α and IL-1β, which is produced from distinct genes and exert similar, although not completely overlapping, biological activities through the IL-1 type I receptor (IL-1RI; CD121a) [3]. In addition, another member of the IL-1 gene family, the IL-1 receptor antagonist (IL-1Ra), binds to IL-1RI without exerting agonistic activity [3].

In the immune system, IL-1 is known as a lymphocyte activating factor [4]. IL-1 acts on monocytes and macrophages to induce production of IL-1, TNFα, IL-6, IL-8, PGE_2 and NO, enhancing their killing activities against bacteria, protozoa, and tumor cells [4]. IL-1 also acts on NK cells in collaboration with IL-2 and IFN-γ to potentiate their cytotoxic activity [4]. Furthermore, it was shown that IL-1 promotes T cell proliferation in response to antigens and lectins, and induces expression of IL-2 and IL-2R [5,6]. IL-1 is involved in the proliferation of Th2 cells synergistically with IL-2 and IL-4 [7], or independently of IL-4 [8], and required for IL-12-induced Th1 cell development [9]. Moreover, IL-1 potentiates γδ T cell proliferation synergistically with IL-7 [10]. IL-1 enhances proliferation and differentiation of B cells synergistically with IL-4 and IL-6, and potentiates Ab production [11]. Although the precise molecular mechanisms of this activation remain largely unknown, IL-1 seems to play an important role in host defense mechanisms against microbes through these effects on immune cells.

When mice were immunized with protein antigens together with IL-1, serum Ab production was enhanced, suggesting that IL-1 has an adjuvant effect [12,13]. It was also reported that IL-1 enhanced Ab production to thymus (T cell) -dependent (TD) and thymus (T cell) -independent (TI) antigens *in vitro* and *in vivo* [14]. On the other hand, when mice were immunized with TD antigen, SRBC, IL-1β potentiated Ab production, while IL-1α suppressed the IL-1β effect [15]. Therefore, it is suggested that there is a distinct role of IL-1α and IL-1β in Ab production. However, administration of anti-IL-1RI Ab or recombinant IL-1Ra during immunization with SRBC or another TD antigen, trinitrophenyl-conjugated keyhole limpet hemocyanin (TNP-KLH), did not affect Ab production [16]. Furthermore, specific serum Ab levels were normal in IL-1RI$^{-/-}$ mice when mice were immunized with TNP-KLH together with alum or complete Freund's adjuvant (CFA) [17,18]. On the other hand, We found that IL-1Ra$^{-/-}$ mice developed chronic inflammatory arthropathy spontaneously and production of autoantibodies against Igs, type II collagen and dsDNA, increased in these mice [19]. Thus, these apparently disparate findings indicate that the

mechanisms of action of IL-1 and individual roles of IL-1α and IL-1β in Ab production still remain to be elucidated.

The current investigation studied the functions of IL-1 in the humoral immune response using IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice. I show that IL-1β, but not IL-1α, is involved in T-dependent Ab production via antigen-specific T cell activation.

Materials and Methods

Mice

IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice were generated by homologous recombination as described previously and backcrossed to BALB/cA mice for 7 or 8 generations [20]. All the 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 8 to 12 week-old adult mice were used for the experiments.

Immunization of mice

Mice were immunized with either 1 × 10^8 SRBC in PBS, 25 μg TNP-Ficoll (kindly provided by Dr. Hideo Nariuchi; Institute of Medical Science, University of Tokyo), or 50 μg TNP-LPS (Paesel + Lorei GmbH&Co, Frankfurt, Germany), intraperitoneally. Primary and secondary responses were examined after immunization with SRBC and TNP-Ficoll, while only the primary response was examined under TNP-LPS immunization. Blood samples were collected from the tail vein before the immunization, and 1 and 2 weeks after the primary immunization. At 15 days after the primary immunization, mice were given the secondary immunization and blood samples were collected 1 and 2 weeks later.

Measurement of Ab titers

Immunoglobulin levels in sera or culture supernatants were measured by ELISA as described previously [21]. To detect SRBC or TNP-specific antibodies, soluble SRBC antigen (2 μg/ml) or TNP-BSA in PBS (10 μg/ml), which was prepared as described previously [22,23], was coated on Falcon 3912 Micro Test III™ Flexible Assay Plates (Becton Dickinson, Oxnard, CA) at 4°C overnight. After incubation, the well was washed with TBS+0.05% Tween 20 for 3 times, and appropriate diluted serum samples were applied and incubated for 1 h at room temperature. After washing, alkaline phosphatase (AP)-conjugated goat anti-mouse IgM and IgG (ZYMED, San Francisco, CA) were added and incubated for 1 h at room temperature. Alkaline phosphatase activity was measured using Substrate Phosphatase SIGMA104® (SIGMA, St. Louis, MO) as the substrate, and the absorbancy at 415 nm is shown.

Preparation of cells from lymphoid tissues

Single cell suspension was prepared from the spleen or lymph nodes (axillary, inguinal and brachial). Spleen cells were treated with a hemolysis buffer (17 mM Tris.HCl, 140 mM NH_4Cl, pH 7.2) to remove red blood cells. Adherent cells and non-adherent cells were separated after incubation for 1 h on a 10 cm dish. Splenic adherent cells (SACs) were collected and used as APCs in the SRBC-specific T cell proliferative response assay. To prepare splenic and lymph node T cells, non-adherent cells were passed through a nylon wool column. T cells were purified by treating the T cell preparation with anti-mouse B220 and anti-mouse Mac-1 magnetic beads (Miltenyi Biotec GmbH) and then passing them through a MACS column. The purity of T cells was monitored by FACScan and was usually about 90%.

B cell proliferative response

Spleen cells (2×10^5 cells/well) were prepared from wild-type or gene-targeted mice and were plated on a 96-well flat-bottom plate with 2 μg/ml of lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI) or 10 μg/ml anti-IgM (cappel, ICN Phamaceuticals, Inc., Aurora, Ohio) in a final volume of 200 μl RPMI1640/10% FCS and cultured for 48 h. After 48 h of culture, [^{3}H]-thymidine (0.25 μCi/ml) (Amercham, Buckinghamshire, England) was added in the culture. After 6 h, cells were harvested using Micro 96 cell harvester (SKATRON, Lier, Norway) and incorporated [3H]-thymidine was measured using Micro Beta System (Pharmacia Biotech, Piscataway, NJ).

T cell proliferative response

For CD3 treatment, splenic T cells or lymph node T cells (2×10^5 cells/well) were plated on a 96-well plate coated with anti-mouse CD3 Ab (1 μg/ml) (145-2C11; PharMingen, San Diego, CA) in the presence or absence of 10 μg/ml of anti-mouse CD28 Ab (PV-1; kind gift by Dr. Ryo Abe; Research Institute for Biological Science, Science University of Tokyo). For SRBC-specific T cell proliferative response assay, T cells were prepared from the spleen 7 to 10 days after the immunization with SRBC. The T cells (5×10^5 cells/well) as well as the irradiated-SACs from non-immunized mice were then co-cultured for 5 days in the presence or absence of soluble SRBC antigen (50 μg/ml). SACs from IL-1$\alpha/\beta^{-/-}$ mice were plated at a concentration of 5×10^4 cells/well. On the other hands, SACs from IL-1Ra$^{-/-}$ mice were plated at 1×10^3 cells/well in 96 well plate. Cell proliferation was measured by the incorporation of [^{3}H] thymidine as described above.

Phagocytotic abilities of APCs

To measure phagocytic activity, 3×10^5 cells of SACs or peritoneal exude cells (PECs) collected by washing the peritoneal cavity with PBS were incubated for 1 h at 37°C with FITC-latex-beads (provided by Dr. Yoshitsugu Matsumoto; School of Agriculture and Life Sciences, University of Tokyo), FITC-Dextran (10 μg/ml; SIGMA) or Lucifer Yellow (2 μg/ml, SIGMA). Then, cells were washed and the fluorescence was analyzed with a FACScan (Becton Dickinson).

Measurement of cytokine levels

The culture supernatant in SRBC-specific T cell response was collected after 2 days (IL-2) or 5 days (other cytokines). IL-2, IL-4, IL-5, IL-6 and GM-CSF levels were determined by Titer Zyme EIA kit (PerSeptive Diagnostics, Inc., Cambridge, MA). As standard recombinant cytokines, mouse IL-2 (Genzyme, Cambridge, MA), IL-4, GM-CSF (PEPROTECH), IL-5 and IL-6 (PerSeptive Diagnostics) were used. IFN-γ levels were determined by an ELISA system using following reagents; a monoclonal hamster anti-mouse IFN-γ (Genzyme) as a capture Ab, a rabbit anti-mouse IFN-γ Ab (provided by Dr. Masayoshi Kohase; Department of Viral Disease and Vaccine Control, National Institute of Health Japan) as the second Ab, AP-conjugated goat anti-rabbit IgG as the third Ab, and Substrate Phosphate SIGMA104® (SIGMA) as the substrate for alkaline phosphatase. A recombinant mouse IFN-γ (SHIONOGI & CO., LTD., Osaka, Japan) was used as a standard. TMB One-Step Substrate System was purchased from DAKO (Carpinteria, CA).

Statistics.

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

Results

Ab production to SRBC in IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice

To evaluate whether or not IL-1 is involved in Ab production, wild-type, IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice were immunized with SRBC intraperitoneally, and SRBC-specific serum Ab levels were measured by enzyme-linked immunosorbent assay (ELISA). Consistent with our previous data [21], SRBC-specific Ab levels of IgM and IgG in IL-1α/β$^{-/-}$ mice were significantly lower than those in wild-type mice after both primary and secondary immunization (Fig. 1A). In contrast, SRBC-specific IgG level in IL-1Ra$^{-/-}$ mice were increased compared with wild-type mice, although IgM levels were comparable in both mice (Fig. 1B). These results clearly show that naturally occuring IL-1 plays important roles in the production of antigen-specific antibodies.

Effects of IL-1-deficiency on intrinsic B cell and T cell function and on phagocytotic abilities of APCs

In order to elucidate the mechanism by which IL-1 activates Ab production, I examined Ab production against TI antigens. It is known that Ab production against TNP-LPS which belongs to a type 1 TI antigen does not require T cell help. When IL-1α/β$^{-/-}$ mice were immunized with TNP-LPS intraperitoneally, Ab production was normal (Fig. 2A), indicating that Ab producing ability of B cells without T cell help is normal in those mice.
To examine the direct effect of IL-1-deficiency on intrinsic B cell functions, B cell responses to LPS and anti-IgM Ab stimulus were investigated. The results showed that the proliferative responses of splenocytes were not different between IL-1α/β$^{-/-}$ and wild-type mice (Fig. 2B). Two to three days after stimulation with anti-IgM Ab, the expression levels of I-A^{d}, CD80 and CD86 on B220^{+} cells were not different among these mice (data not shown). These results indicate that the lack of IL-1 does not have any direct effect on B cell function.

I next analyzed the intrinsic functions of T cells in IL-1α/β$^{-/-}$ mice. When splenic T cells from IL-1α/β$^{-/-}$ mice were stimulated with immobilized anti-CD3 Ab or immobilized anti-CD3 Ab plus soluble anti-CD28 Ab, proliferative responses of these cells were normal (Fig. 3A). IL-4 and IFN-γ productions after stimulation with immobilized anti-CD3 Ab were normal in IL-1α/β$^{-/-}$ mice under these conditions (Fig. 3B). Thus, it was shown that intrinsic T cell function was not affected by the deficiency of IL-1, so far as it was estimated by these tests.

Next, I examined phagocytic activity of macrophages from IL-1α/β$^{-/-}$ mice. As

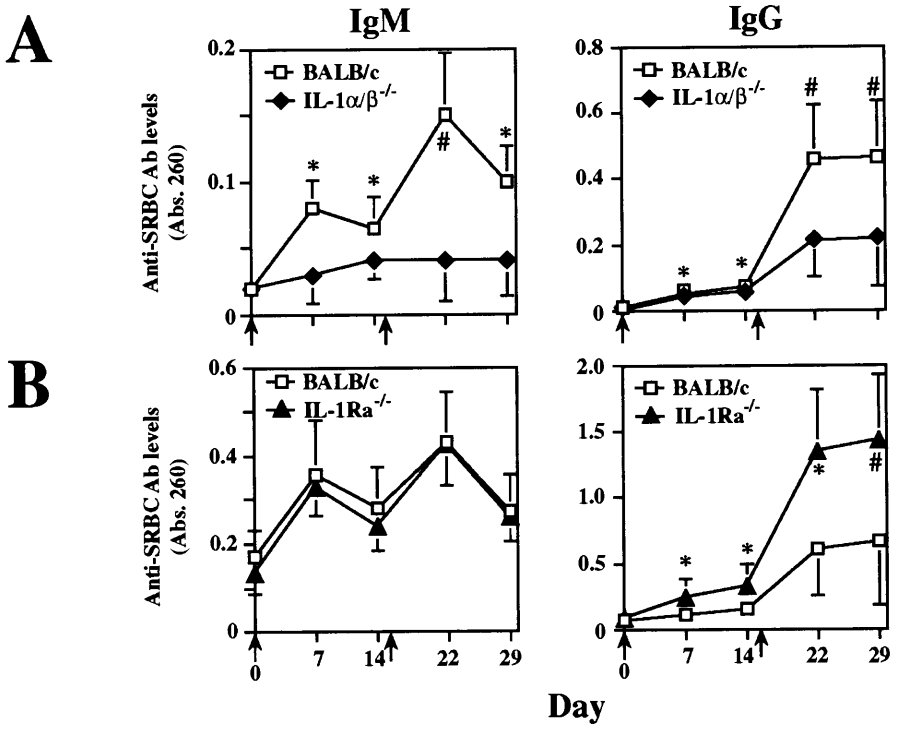

Figure 1. Efficiency of Ab production against SRBC in IL-1α/β-/- and IL-1Ra-/- mice
Mice were immunized with SRBC (arrow point), and sera were collected 1 and 2 weeks after the primary and secondary immunization. After appropriate dilution of the serum (IgM and IgG: 1/100 dilution), SRBC-specific Ab levels in the sera were measured by ELISA. (A) wild-type mice: n=10, IL-1α/β-/- mice: n=10. (b) IL-1Ra-/- mice: n=7. The average and standard deviation (SD) are shown. * $p<0.05$, and # $p<0.005$.

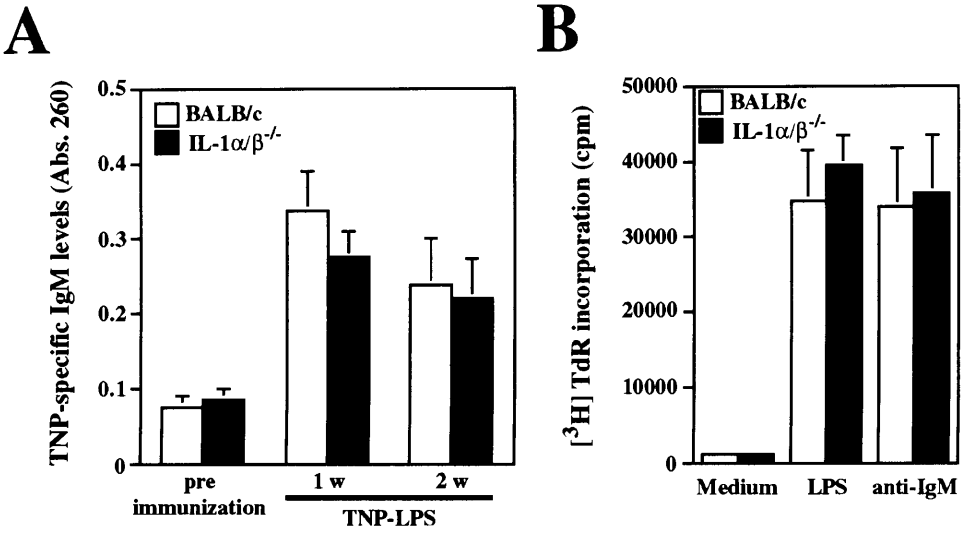

Figure 2. Intrinsic functions of B cells in IL-1a/b-/- mice
(A) Mice were immunized with either TNP-LPS, and the sera were collected before immunization, and 1 and 2 weeks after immunization. After 1/1000 dilution, anti-TNP--specific IgM levels in sera were measured by ELISA. Wild-type mice: n=4, IL-1α/β-/- mice: n=4. The average and SD are shown. (B) Proliferative responses of splenocytes against mitogenic stimulations were measured by the incorporation of [3H] thymidine using IL-1α/β-/- mice. Bars represent an average of 2-3 mice with SD. One representative result from three independent experiments is shown.

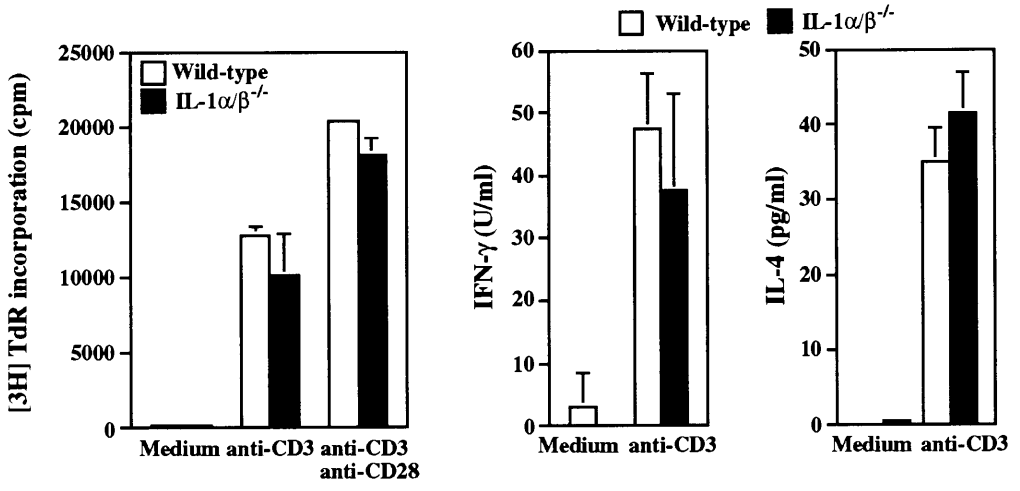

Figure 3. Intrinsic functions of T cells in IL-1α/β-/- mice
Proliferative responses of splenic T cells against plate-coated anti-CD3 mAb only or with soluble anti-CD28 mAb were measured by the incorporation of [3H] thymidine using IL-1α/β-/- mice (A). Cytokine levels in culture supernatants were measured by ELISA (B). Bars represent an average of 2-3 mice with SD. One representative result from three independent experiments is shown.

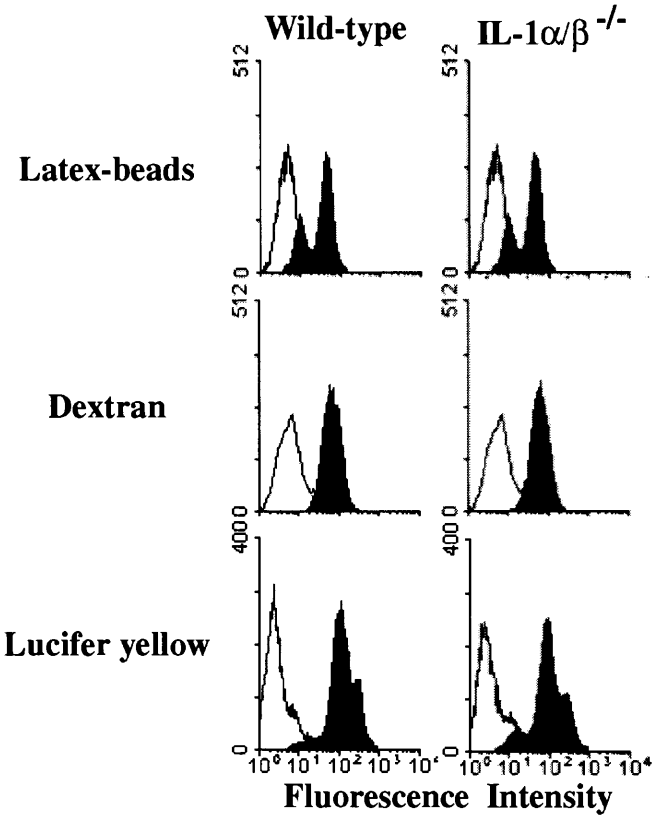

Figure 4. Phagocytotic abilities of APCs of IL-1α/β-/- mice
Phagocytic abilities for FITC-latex beads and FITC-dextran of PECs and pinocytic abilities for lucifer yellow of SACs from wild-type and IL-1α/β-/- mice were assessed by flow cytometry. One representative result from at least three independent experiments is shown.

shown in Figure 4E, phagocytic activities of the peritoneal macrophages to both FITC-latex beads and FITC-dextran were normal (Fig. 4). Pinocytotic activity of splenic adherent cells (SACs) from IL-1$\alpha/\beta^{-/-}$ mice to lucifer yellow was also normal. These results suggest that IL-1 is neither involved in the phagocytic activity of APCs, nor in T and B cell intrinsic functions.

The role of IL-1 in T cell-APC interaction

I then examined the role of IL-1 in activating antigen-specific T cell helper function upon interaction with APCs. As shown in Fig. 5A, when splenic T cells from IL-1$\alpha/\beta^{-/-}$ mice immunized with SRBC (T[$\Delta\alpha/\beta$]) and APCs from non-immunized wild-type mice (APC[W.T.]) as well as in the (T[WT]+APC[$\Delta\alpha/\beta$]) culture were co-cultured in the presence of soluble SRBC antigens, the T cell proliferative response decreased by 1/3 of that observed in (T[WT]+APC[WT]) culture. The response in the (T[$\Delta\alpha/\beta$]+APC[$\Delta\alpha/\beta$]) co-culture was most severely impaired among these combinations (T[WT]+APC[WT]: 100% vs. T[WT]+APC[$\Delta\alpha/\beta$]: 57% ± 21%, $p < 0.005$; T[$\Delta\alpha/\beta$+ APC[WT]: 63% ± 19%, $p < 0.005$; T[$\Delta\alpha/\beta$]+APC[$\Delta\alpha/\beta$]: 19% ± 16%, $p < 0.0005$, respectively. Average ± SD after subtraction of the background response in the absence of antigen from six independent experiments is shown). The (T[$\Delta\alpha/\beta$]+APC[$\Delta\alpha/\beta$]) culture also showed a markedly decreased production of cytokines, such as IL-2, IL-4, IL-5, IL-6, GM-CSF, and IFN-γ (Fig. 5B). On the other hand, co-culture of splenic T cells from IL-1Ra$^{-/-}$ mice immunized with SRBC (T[Δra]) and APCs from non-immunized IL-1Ra$^{-/-}$ mice (APC[Δra]) in the presence of soluble SRBC antigens showed the highest response (Fig. 5C) (T[WT]+APC[WT]: 100% vs. T[WT]+APC[Δra]: 147% ± 18%, $p < 0.05$; T[Δra]+ APC[WT]: 417% ± 255%, $p < 0.05$; T[Δra]+APC[Δra]: 901% ± 509%, $p < 0.05$, respectively. Average ± SD after subtraction of the background response in the absence of antigen from four independent experiments). In addition, IL-2, IL-4, IL-5, IL-6, and GM-CSF levels in the (T[Δra]+APC[Δra]) culture fluid were increased compared with that in the (T[WT]+APC[WT]) culture fluid (Fig. 5D). In contrast, the IFN-γ level in the (T[Δra]+APC[Δra]) culture fluid was lower than that in the (T[WT]+APC[WT]) culture fluid (Fig. 5D). IL-1α, IL-1β and TNFα could not be detected in those culture fluids (data not shown). It is known that wild-type APCs produce IL-1 and IL-1Ra. However, the change of T cell response by IL-1-deficiency or IL-1Ra-deficiency was not ameliorated by the co-culture with wild-type APCs. These results suggest that IL-1 is involved in the induction of antigen-specific memory T cells in T cell-priming *in vivo*. These results indicate that IL-1 is involved in the activation of T cells through interaction with APCs,

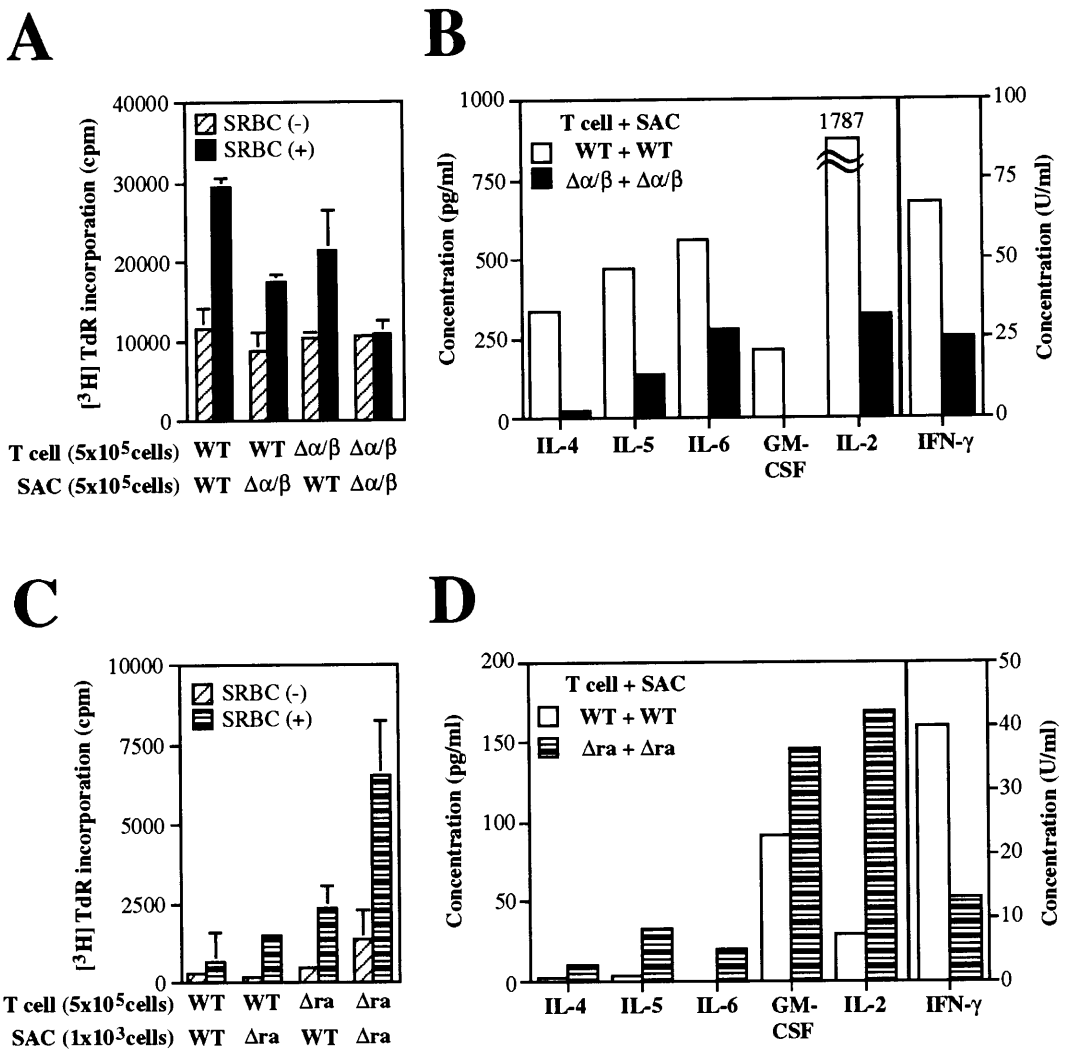

Figure 5. Impaired T cell responses through T-APC interaction in IL-1α/β-/- mice
Proliferative responses and cytokine production of antigen-specific T cells upon interaction with SACs were determined. T cells (2 x 105 cells) from the spleen of SRBC-immunized wild-type (WT), IL-1α/β-/- (Δα/β) (A), and IL-1Ra-/- (Δra) mice (C) were cultured with SACs from non-immunized mice in absence (hatched column) or presence (closed column) of soluble SRBC antigen, and the proliferative responses were measured by the incorporation of [3H] thymidine after 5 days. Cytokine levels in the supernatants of the IL-1α/β-/- mouse (B) or IL-1Ra-/- mouse (D) T cell culture were measured. Average ± SD is shown in (A) and (C), while bars in (B) and (D) show the total amount of the cytokine in triplicate culture supernatants. One representative result from three independent experiments is shown.

suggesting that the defect of Ab production in IL-1$\alpha/\beta^{-/-}$ mice may be caused by the reduced responses of antigen-specific T cells.

Ab production to SRBC in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice

It was suggested that IL-1α and IL-1β play distinct roles in Ab production [15]. When Ab production was examined in either IL-1$\alpha^{-/-}$ or IL-1$\beta^{-/-}$ mice, SRBC-specific IgM and IgG levels in IL-1$\beta^{-/-}$ mice were clearly lower than those in wild-type mice, whereas those in IL-1$\alpha^{-/-}$ mice were normal (Fig. 6A and B). These results indicate that IL-1β, but not IL-1α, plays a major role in T cell-dependent Ab production.

Ab production to type 2 thymus-independent (TI) antigens in IL-1$\alpha/\beta^{-/-}$ mice

It is known that Ab production against TNP-LPS which belongs to a type 1 TI antigen does not require T cell help, while that to type 2 TI antigens such as TNP-Ficoll still requires T cell help, although the mechanism of the humoral immune response to type 2 TI antigens is considered to be different from that to TD antigens [24]. When IL-1$\alpha/\beta^{-/-}$ mice were immunized with TNP-Ficoll intraperitoneally, TNP-specific IgM and IgG levels were significantly reduced in IL-1$\alpha/\beta^{-/-}$ mice both after primary and secondary immunization (Fig. 7). This reduction was seen in all the IgG subclasses (data not shown). These results indicate that IL-1 is also involved in the Ab production to type 2 TI antigens.

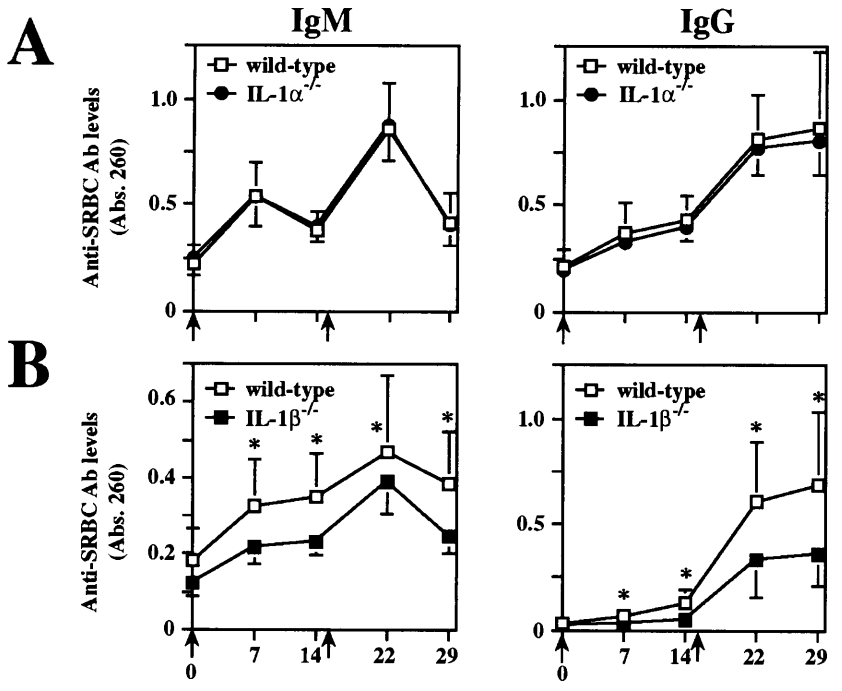

Figure 6. Efficiency of Ab production against SRBC in IL-1α-/- and IL-1β-/- mice
Mice were immunized with SRBC (arrow point), and sera were collected 1 and 2 weeks after the primary and secondary immunization. After appropriate dilution of the serum (IgM and IgG: 1/100 dilution), SRBC-specific Ab levels in the sera were measured by ELISA. (A) wild-type mice: n=8, IL-1α-/- mice: n=7. (B) IL-1β-/- mice: n=7. The average and standard deviation (SD) are shown. * $p<0.05$, and # $p<0.005$.

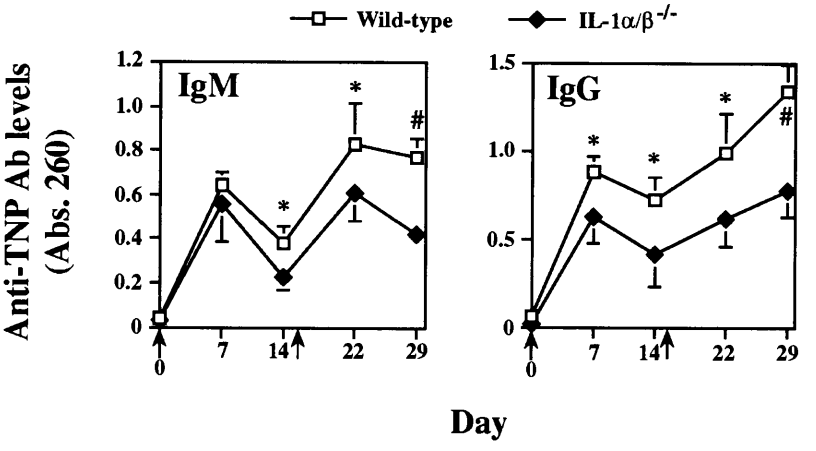

Figure 7. Ab production against tytpe2 TI antigens in IL-1α/β-/- mice
Mice were immunized with TNP-Ficoll (arrow point), and the sera were collected 1 and 2 weeks after the primary and secondary immunization. After 1/1000 dilution, anti-TNP-specific immunoglobulin levels in sera were measured by ELISA. Wild-type mice: n=5, IL-1α/β-/- mice: n=5. The average and SD are shown. * $p<0.05$, and # $p<0.005$.

Discussion

In the previous studies, adjuvant effects of exogenous IL-1 were shown in humoral immune responses [12,13], however, so far the roles of endogenous IL-1 have not been elucidated clearly. The effects of IL-1RI-deficiency were not observed in the humoral immune responses against TNP-KLH when mice were immunized with alum or complete Freund's adjuvant (CFA) [17,18]. Furthermore, administration of anti-IL-1RI Ab or recombinant IL-1Ra during immunization with SRBC or TNP-KLH did not affect the Ab production [16]. In this report, I have first shown that Ab production against SRBC was severely reduced in IL-1$\alpha/\beta^{-/-}$ mice, showing that endogenous IL-1 plays a crucial role in the humoral immune response under the physiological conditions. This apparent discrepancy seems to be caused by the difference of the immunization protocols; they used CFA in their experiments, while I immunized without adjuvant. Probably, immunization with adjuvant may induce various inflammatory cytokines that substitute for the function of IL-1. Consistently with this notion, I found that Ab production after immunization with SRBC without adjuvant was reduced in IL-1RI$^{-/-}$ mice (Nakae et al., unpublished data). Mycobacteria in CFA also affect the development of collagen-induced arthritis (CIA) by stimulating expansion of the Mac-1^{+} cell population [25], and IFN-γ causes apparently opposite effects on the development of the arthritis depending on the presence of CFA. Enhancing effects of CFA on antibody production were also observed in CD80/CD86$^{-/-}$ and ICOS$^{-/-}$ mice [26,27], in which the strong inflammatory response elicited by CFA immunization induced CD80 and CD86 expression which compensated the defects of co-signaling molecules.

The immune cell compartment of the thymus, spleen and lymph nodes, as well as the splenic microarchitecture was normal in IL-1$\alpha/\beta^{-/-}$ mice (data not shown). Germinal center formation was also normally observed in the mutant mice (data not shown). Moreover, intrinsic functions of B cells and T cells from IL-1$\alpha/\beta^{-/-}$ mice were normal. However, SRBC-specific proliferative response and Th1/Th2 cytokine production of T cells through interaction with APCs were markedly impaired in IL-1$\alpha/\beta^{-/-}$ mice, and enhanced in IL-1Ra$^{-/-}$ mice. These observations suggest that T cell-APC interaction is impaired in these IL-1 mutant mice. In this context, recently, I demonstrated that IL-1 is involved in the T cell activation during the primary T cell response through interaction with APCs [21]. Therefore, the defect of secondary SRBC-specific T cell response in IL-1$\alpha/\beta^{-/-}$ mice is considered to be caused by the failure of T cell-priming and antigen-specific memory T cell-induction.

It is remarkable that the antibody production was reduced only in IL-1$\beta^{-/-}$ mice, but normal in IL-1$\alpha^{-/-}$ mice, indicating that two IL-1 molecules play distinct roles in the immune

system. With regard to this, it was reported that rIL-1β as well as a nonapeptide derived from the IL-1β sequence potentiated antigen-specific Ab production when mice were immunized with SRBC, while rIL-1α suppressed the rIL-1β action [14,15]. However, I showed that Ab levels in wild-type and IL-1α$^{-/-}$ mice were similar, indicating that endogenous IL-1α is not inhibitory to the Ab production. Probably, the concentration of rIL-1α was too high in their experiments compared to that of the physiological IL-1α, causing non-specific suppression of the immune reaction.

The mechanism for this functional discrimination between IL-1α and IL-1β is not known completely, however, some possibilities are conceivable. It is known that T cell priming occurs in PALS in LNs or the T cell zone in the spleen through interaction with dendritic cells (DC). It was reported in studies of human dentritic cell (DC) that monocyte-derived DC, termed DC1, synthesized IL-1α but DC2, derived from plasmacytoid cells, did not [28]. Furthermore, it was also reported that CD11a^{-}CC81^{-}MyD-1^{+} DC from bovine afferent lymph synthesized IL-1α and simulated both CD4^{+} and CD8^{+} T cells, while CD11a^{+}CC81^{+}MyD-1^{-} DC that did not synthesize IL-1α could not stimulate CD8^{+} T cells [29]. Thus, it is possible that CD4^{+} T cells that are involved in the humoral immune response are stimulated by a particular subpopulation of DC that synthesizes only IL-1β. As another possibility, this discrimination may occur at the B cell maturation steps, because it was reported that IL-1β, but not IL-1α, is strongly expressed in follicular dendritic cells in the germinal center that plays important roles in affinity maturation and isotype switch of Igs through interaction with B cells [25]. On the other hand, I previously reported that IL-1α and IL-1β are mutually inductive, and IL-1α expression is particularly dependent on IL-1β expression [20]. Thus, it is also possible that IL-1α-deficiency is compensated by IL-1β, while IL-1β-deficiency is not, causing detrimental effects on the immune system.

I showed that IFN-γ levels in the culture supernatants of T cells stimulated with SRBC antigens were reduced both in IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice. Since IL-1 is required for the development of antigen-specific T cell priming and memory T cell induction [21,30], memory T cells may develop only poorly after the first immunization in the absence of IL-1. I think this is the reason why cytokine production both of Th1/Th2 classes was reduced in IL-1-deficient mice upon secondary immunization. On the other hand, a defect of IL-1Ra would cause an excess IL-1 signal to the cells, because IL-1Ra suppresses both IL-1α and IL-1β activity. Thus, the enhancement of IL-4, IL-5, and IL-6 production and reduction of IFN-γ production suggests that IL-1 promotes development of Th2 population. In consistent with this observation, I found that IL-4 production was enhanced in IL-1Ra$^{-/-}$ mice on a BALB/c background upon *Nippostrongylus brasiliensis* infection (Nakae et al., unpublished data). However, in contrast to these observations, it was reported that IL-1 promoted Th1

responses in mice on the BALB/c background, but not on the C57BL/6 background [9]. It was also reported that Th2 cytokine production was enhanced in IL-1RI$^{-/-}$ mice infected with *Leishmania major* and immunized with KLH/CFA [18]. Therefore, the effects of IL-1-deficiency and excessive IL-1 signaling on Th1/Th2 balance seem complex. I are now further analyzing the role of IL-1 in Th1/Th2 differentiation using the *Nippostrongylus brasiliensis* infection and Schistosoma egg injection systems (Th2 response) as well as the BCG infection system (Th1).

I found that IgG production specific to SRBC was enhanced in IL-1Ra$^{-/-}$ mice, indicating that excess IL-1 signal accelerates the immune response. One exception was the effects on IgM production; IL-1-deficiency reduced IgM production, whereas IL-1Ra-deficiency did not affect this at all, showing that excess IL-1 signal alone cannot enhance IgM production.

I also found that TNP-specific Ab production of both IgM and IgG subclasses was markedly reduced in IL-1$\alpha/\beta^{-/-}$ mice when those mice were immunized with TNP-Ficoll. Although Ab production to type 2 TI antigens requires T cell help, the mechanism of this function is thought to be different from that of TD antigens [24]. Recently, I have shown that IL-1 enhances T-dependent Ab production through induction of CD40L and OX40 on T cells [21]. It is known, however, that the Ab production against type 2 TI antigens is neither dependent on interactions between TCR and MHC class II, CD28 and CD80/CD86, CD40L and CD40, nor OX40 and OX40L [31-35]. Thus, the mechanisms in which IL-1 is involved may be different between responses against type 2 TI antigens and TD antigens.

In conclusion, these observations indicate that IL-1β, but not IL-1α, is a potent activator of the humoral immune response, and that IL-1Ra has important regulatory functions in the immune system. Immune enhancing activity of IL-1 should be of benefit for the host defense mechanism, because IL-1 produced upon infection with bacteria or viruses would enhance the immune response against these pathogens.

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CHAPTER III

IL-1α, but not IL-1β, Is Required for Contact-Allergen-Specific T Cell Activation during the Sensitization Phase in Contact Hypersensitivity

Summary

Contact hypersensitivity (CHS) is a T cell-mediated cellular immune response caused by epicutaneous exposure to contact allergens. In this reaction, after the first epicutaneous allergen sensitization, Langerhans cells (LCs) catch allergens and migrate from the skin to draining lymph nodes (LNs) and activate naive T cells. Although IL-1 is suggested to be involved in these processes, the mechanisms have not been elucidated completely. In this report, to elucidate roles of IL-1α and IL-1β in CHS, I analyzed ear swelling in 2, 4, 6-trinitrochlorobenzene (TNCB)-induced CHS using gene-targeted mice. I found that ear swelling was suppressed in IL-1α-deficient (IL-1$\alpha^{-/-}$) mice but not in IL-1$\beta^{-/-}$ mice. LC migration from the skin into LNs was delayed in both IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice, suggesting that this defect was not the direct cause for the reduced CHS in these mice. However, I found that the proliferative response of trinitrophenyl (TNP)-specific T cells after sensitization with TNCB was specifically reduced in IL-1$\alpha^{-/-}$ mice. Furthermore, adoptive transfer of TNP-conjugated IL-1-deficient epidermal cells into wild-type mice indicated that only IL-1α, but not IL-1β, produced by antigen-presenting cells in epidermal cells could prime allergen-specific T cells. These observations indicate that IL-1α, but not IL-1β, plays a crucial role in TNCB-induced CHS by sensitizing TNP-specific T cells.

Introduction

Contact hypersensitivity (CHS) is a T cell-mediated cellular immune response caused by repeated epicutaneous exposure against contact allergens, chemically reactive haptens which are able to bind directly to soluble or cell-associated proteins. This response is divided into two phases, sensitization and elicitation phases. After the first epicutaneous allergen sensitization, Langerhans cells (LCs) catch allergens and migrate from the skin to draining lymph nodes (LNs) where naive T cells are thought to be primed against allergens through T cell-LC interaction [1]. In the elicitation phase, allergen-specific T cells in LNs are activated upon re-challenging with the same allergen, and migrate from LNs to the place where the allergen is challenged, resulting in local inflammation. Using MHC class $I^{-/-}$ and MHC class $II^{-/-}$ mice, it was shown that $CD8^+$ T cells act as effector cells, while $CD4^+$ T cells play a regulatory role in this reaction [2,3]. It is known that LCs are important as APCs in the sensitization phase, but they are not essential for the elicitation phase [4].

Both IL-1α and IL-1β cause inflammation and also augment immune reactions through activation of lymphocytes, although they are encoded by distinct genes and have little amino acid sequence homology [5]. The expression of proinflammatory cytokine mRNAs including these of IL-1α, IL-1β and TNFα is increased in the contact allergen-sensitized skin [6]. In the epidermis, IL-1α is mainly produced by kelatinocyts (KCs) while IL-1β by LCs [7-9]. IL-1β mRNA is expressed earlier than any other proinflamatory cytokines in CHS [9,10].

It was shown that systemic administration of recombinant IL-1α (rIL-1α) or local treatment of rIL-1β causes activation and migration of LCs [10-12]. LC migration was impaired by systemic administration of anti-IL-1β antibody [13]. Furthermore, treatment with IL-1 receptor antagonist (IL-1Ra), which is a negative regulator of IL-1α and IL-1β, abolished the enhanced migration of LCs [14,15], and corneal LCs migration was impaired in mice deficient in the IL-1 receptor type 1 (IL-1RI) gene [16]. These observations suggest that both IL-1α and

IL-1β can promote LC migration that may be important in CHS. In consistent with these observations, CHS was markedly reduced by the intradermal administration with anti-IL-1β mAb [10]. However, it was reported that CHS was not affected by the treatment with anti-IL-1α mAb [10]. On the other hand, it was shown that IL-1β was not involved in oxazolone-induced CHS using IL-1β$^{-/-}$ mice [17,18]. Furthermore, it was shown that low dose TNCB-induced CHS was suppressed in IL-1β$^{-/-}$ mice, whereas high dose response was not [18]. Thus, these apparently controversial findings claim that the role of IL-1 in CHS still remains to be elucidated.

CHS develops through several distinct steps including LC migration from the skin into LNs, allergen-specific T cell activation and cell infiltration into inflammed regions. It is suggested that various cytokines, chemokines, adhesion molecules and co-signal molecules are involved in these processes [19-27]. Recently, I have shown that IL-1 produced by antigen-presenting cells (APCs) play a crucial role in antigen-specific T cell-priming and clonal expansion using IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice [28]. In this study I tried to elucidate roles of IL-1 in CHS using these IL-1$^{-/-}$ mice. I found that TNCB-induced CHS at both low and high doses was greatly reduced in IL-1α$^{-/-}$ and IL-1α/β$^{-/-}$ mice, but not in IL-1β$^{-/-}$ mice. The induction of TNP-specific T cell was abolished in IL-1α$^{-/-}$ and IL-1α/β$^{-/-}$ mice, whereas that in IL-1β$^{-/-}$ mice was normal, indicating that IL-1α produced by mature LCs in LNs is required to prime naive T cells against contact-allergens in the sensitization phase of CHS.

Materials and Methods

Mice

IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$ and IL-1$\alpha/\beta^{-/-}$ mice were generated by homologous recombination as described previously and backcrossed to C57BL/6J mice or BALB/cA for 8 generations [29]. DO11.10 Tg mice (BALB/cA background) were kindly provided by Dr. Dennis Y. Loh [30]. All the 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 adult mice of 8 to 12 week-old were used for the experiments.

Contact hypersensitivity response

2, 4, 6-trinitrochlorobenzene (TNCB; Tokyo Kasei, Tokyo, Japan)-induced CHS was assayed as described previously [17,18]. Briefly, abdomen of mice was shaved and sensitized epicutaneously with 25 μl of low dose (0.3%) or high dose (3.0%) TNCB dissolved in acetone and olive oil mixture (4:1). On day 5 after sensitization, the outside of one ear (auricle) of mice was challenged with 25 μl of 1.0% TNCB and the outside of another ear was applied with 25 μl of vehicle alone. At 24 h after the second challenge, mice were euthanized and a disc of ear tissue was removed from both ears using a 6 mm biopsy punch, then each of ear disc was weighed. The difference between TNCB-treated and vehicle-treated ear weights of each mouse is shown as the amount of swelling in TNCB-induced CHS. Ear swelling is calculated as follows; [Increment of ear swelling] = ([weight of challenged ear] - [weight of vehicle-treated ear]) / [weight of vehicle-treated ear] x 100 (%).

Migration and maturation of LCs

Mice were shaved at dorsal and abdominal area, and painted with 50 μl of 0.5% fluorescein isothiocyanate (FITC) isomer I (SIGMA) dissolved in acetone and dibutylphthalate mixture (1:1). At 24 h after FITC painting, inguinal, axillary and brachial LNs were harvested and pooled. Single cell suspension was prepared from collagenase-treated LNs and stained with biotinylated anti-mouse CD11c mAb (HL3; PharMingen, San Diego, CA) after pre-incubation with anti-Fc receptor mAb (2.4G2; PharMingen). To assess the maturation of LCs, LN cells were stained with PE-anti-mouse CD40 mAb (3.23; Immunotech, Marseille Cerdex, France) and PerCP-streptavidin (PharMingen). The frequency of $CD11c^+$ $FITC^+$ cells or expression levels of CD40 on $CD11c^+$ $FITC^+$ cells in LNs were analyzed with FACScan (Beckton Dickinson, Mountain View, CA) using Lysis II software (Beckton Dickinson). Viable cells were determined by forword and side scatters.

Intracellular staining of IL-1α in PECs and mature LCs

Thioglycolate (TGC)-induced peritoneal exudate cells (PECs) were prepared as described previously [29]. LN cells from FITC-painted mice (at 24 h after painting) and PECs were harvested and stimulated with 5 μg/ml of LPS for 6 h, then, cells were suspended in a staining buffer (HANKS containing 2% fetal calf serum and 0.1% sodium azide). After blocking with anti-FcγRII/III receptor mAb (2.4G2; Pharmingen), PECs were treated with FITC-anti-mouse Mac-1 mAb and LN cells from FITC-painted mice were treated with biotinylated anti-CD11c mAb (HL3; PharMingen). Then, cells were incubated with PerCP-streptavidin (PharMingen), followed by fixation with PBS containing 4% paraformaldehyde for 20 min. After washing with a permeabilization buffer (0.1% saponin (SIGMA) in the staining buffer), cells were incubated with PE hamster anti-mouse IL-1α mAb (ALF-161, PharMingen) or isotype-matched control mAb (PE-hamster IgG; Immunotech, Marseille Cerdex, France) in the permeabilization buffer for 30 min at 4℃. Cells were washed with the permeabilization buffer and analyzed using a FACS calibur (Beckton Dickinson) and Cell

Quest software (Beckton Dickinson).

T cell proliferative response assay

For TNP-specific T cell proliferative response, inguinal, axillary and brachial LNs were harvested and pooled 5 days after the sensitization with 3.0% TNCB. Single cell suspension was prepared, and T cells were purified by passing through MACS separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to remove anti-B220 and anti-Mac-1 mAb reactive cells. To prepare TNP-conjugated APCs, the spleen was harvested from wild-type mice and single cell suspension was prepared. After treatment with hemolysis buffer (17 mM Tris.HCl, 140 mM NH_4Cl, pH 7.2), T cells were depleted by passing through MACS columns using anti-Thy1.2, anti-CD4 and anti-CD8 magnetic beads (Miltenyi Biotec GmbH). T cell-depleted spleen cells were incubated in PBS containing 100 mM trinitrobenzene sulfonate (TNBS; WAKO, Osaka, Japan) at 37°C for 5 min, and irradiated with γ-ray (3,500 rad). LN T cells from TNCB-sensitized mice (5×10^5 cells/well) and TNP-conjugated APCs (2×10^5 cells/well) were cultured in 200 μl of 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% heat-inactivated fetal calf serum (FCS) (SIGMA) using 96 well flat-bottom plate for 72 h. For OVA-specific primary T cell proliferative responses, inguinal, axillary and brachial LNs of DO11.10 Tg mice were incubated with anti-B220, anti-Mac-1 and anti-CD8 magnetic beads, and $CD4^+$ T cells were purified by passing through MACS column. To collect skin LCs, ear skin was incubated with 0.15 % trypsin (GIBCO) and 50 U/ml of dispase (Godoshusei) in PBS for 1 h at 37 °C, and epidermal sheets were prepared. Single cell suspension from epidermal sheets was prepared, and $CD11c^+$ LCs were isolated with MACS system by positive selection using biotinylated anti-CD11c and streptavidin-magnetic beads (Miltenyi Biotec GmbH). DO11.10 $CD4^+$ T cells (5×10^5 cells/well) were cultured with TGC-induced PECs or $CD11c^+$ skin LCs (5×10^3 cells/well) in

the absence or presence of the OVA peptide (0.1 μM) with or without rIL-1α (100 pg/ml) for 72 h. After 72 h, cells were labeled with [^{3}H]-thymidine (0.25 μCi/ml) (Amercham, Buckinghamshire, England) for 6 h, then, were harvested using Micro 96 cell harvester (SKATRON, Lier, Norway) and [^{3}H] radioactivity was measured using Micro Beta System (Pharmacia Biotech, Piscataway, NJ).

Preparation of epidermal cells

Mice were shaved at dorsal and abdominal area and remove the hair completely with a hair-remover cream at 2 days before experiments. The hair-removed skin were harvested and removed the hypodermal tissue. The skin was incubated with 0.15 % trypsin (GIBCO) and 50 U/ml of dispase (Godoshusei) in PBS for 1 h at 37 °C, and epidermal sheets were prepared. Epidermal sheets were stirred in PBS containing 2% FCS for 15 min at room temperature. EC suspension was obtained by filtrating epidermal sheet suspension with the nylon mesh. TNP-conjugated epidermal cells (ECs) were prepared by incubating the cell suspension with 100 mM TNBS for 10 min at 37 °C as described above.

Adoptive transfer of TNP-conjugated splenocytes and induction of CHS

TNP-conjugated ECs from wild-type or IL-1-deficient mice were suspended in PBS, and injected into wild-type mice subcutaneously ($2x10^6$ cells/mouse). At 7 days after injection, the outside of one ear of mice was challenged with 25 μl of 1.0% TNCB and the outside of another ear was applied with 25 μl of vehicle alone. At 24 h after the challenge, ear swelling was measured as described above.

Measurement of Ab titers

Mice were sensitized and challenged with TNCB as described above. Four days after the challenge, the sera were collected. TNP-specific immunoglobulin levels in the sera were measured by sandwitch enzymed-linked immunosorbent assay (ELISA). To TNP-specific

antibodies, TNP-BSA in PBS (10 μg/ml) was coated on Falcon 3912 Micro Test III™ Flexible Assay Plates (Becton Dickinson, Oxnard, CA) at 37 °C overnight. After washing with TBS, serial diluted serum samples were applied and incubated at room temperature for 1 h. After incubation 1 h the well was washed with TBS+0.05% Tween 20, followed by the addition of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (ZYMED, San Francisco, CA). Alkaline phosphatase activity was measured using Substrate Phosphatase SIGMA104® (SIGMA, St. Louis, MO) as the substrate. Results are expressed by the absorbancy at 415 nm.

Statistics

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

Results

CHS with high dose of TNCB

It was previously reported that CHS was markedly reduced in IL-1β$^{-/-}$ mice when mice were sensitized with low dose of TNCB, whereas high dose sensitization induced comparable CHS between IL-1β$^{-/-}$ and wild-type mice [17]. These results suggest that IL-1β-deficiency can be compensated by IL-1α at high dose TNCB sensitization, but not at low dose sensitization. In order to know the differential roles of IL-1α and IL-1β, I reexamined CHS at both low and high TNCB doses using IL-1α$^{-/-}$, IL-1β$^{-/-}$ and IL-1α/β$^{-/-}$ mice on the C57BL/6J background (Fig. 1A and B). In contrast to the previous report, I found that similar levels of CHS were induced in IL-1β$^{-/-}$ and wild-mice with both low and high doses of TNCB. Furthermore, I found that CHS was significantly reduced in IL-1α$^{-/-}$ mice as well as IL-1α/β$^{-/-}$ mice at 24 h after the challenge (Fig. 1A and B). In our assay system, ear inflammation in wild-type and IL-1$^{-/-}$ mice was calmed down to the basal levels at 48 h after the challenge (data not shown). I assessed the effect of genetic background using IL-1-deficient mice on the BALB/cA background, because genetic background affects on CHS [31]. Similar results were obtained using IL-1α/β$^{-/-}$ and IL-1β$^{-/-}$ mice on the BALB/cA background (data not shown). However, the effect of IL-1α-deficiency was only small on the BALB/cA background compared to that on the C57BL/6J background. Since I observed more pronounced effects of the deficiency in IL-1α/β$^{-/-}$ mice compared with IL-1α$^{-/-}$ mice on the BALB/cA background, it was suggested that IL-1β also play some role in a synergistic manner with IL-1α in this background mice.

Effects of IL-1-deficiency on LC migration and maturation

To elucidate roles of IL-1 in CHS, I first examined migration ability of LCs from the skin into draining LNs. After sensitization with 0.5% FITC, CD11c^{+} FITC^{+} cell counts in draining LNs were measured by flow cytometry analysis (Fig. 2A). The content of CD11c^{+}

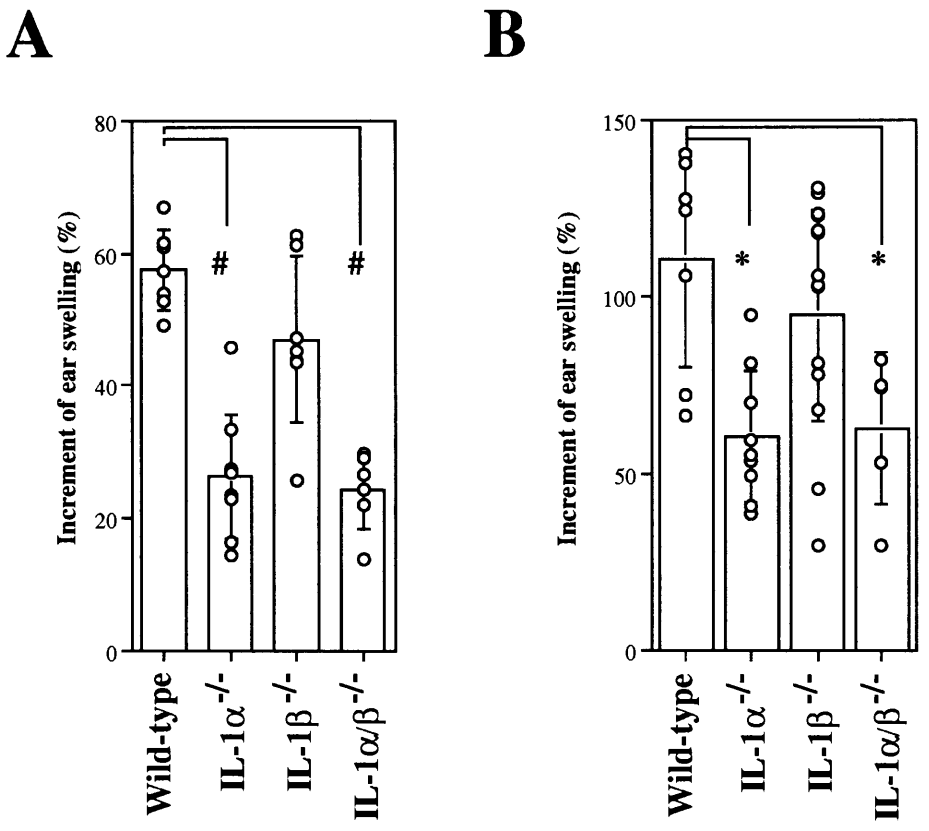

Figure 1. TNCB-induced CHS in wild-type, IL-1α-/-, IL-1β-/- and IL-1α/β-/- mice. Increment of ear swelling in TNCB-induced CHS. (A) Low dose (0.3%) TNCB-induced CHS in wild-type (n=7), IL-1α-/- (n=8), IL-1β-/- (n=7) and IL-1α/β-/- (n=6) mice. (B) High dose (3.0%) TNCB-induced CHS in wild-type (n=8), IL-1α-/- (n=10), IL-1β-/- (n=14) and IL-1α/β-/- (n=6) mice. Each circle represents an individual mouse and an average and standard deviation (SD) are shown. * $p < 0.01$ and # $p < 0.001$

FITC$^+$ cells in LNs from both IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, and IL-1$\alpha/\beta^{-/-}$ mice was significantly reduced compared with wild-type mice after FITC-treatment. The migration in IL-1$\alpha/\beta^{-/-}$ mice was most severely affected among them at 24 h after the treatment. However, the FITC$^+$ LC content in LNs of both IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice became similar to that of wild-type mice at 36 hrs after FITC-treatment (Fig. 2B).

It is known that skin LCs are immature as dendritic cells and they maturate during migration from the skin into draining LNs. To examine whether IL-1 is involved in LC maturation, expression levels of CD40 on CD11c$^+$ FITC$^+$ cells were examined by flow cytometry analysis (Fig. 2C), because CD40 is expressed on mature LCs, but not on immature LCs [32]. Comparable expression levels of CD40 were observed on FITC$^+$ cells from IL-1-deficient and wild-type mice. Furthermore, I could not detect any difference in the expression levels of CD80, CD86 and CD54 on CD11c$^+$ FITC$^+$ cells from these mice (data not shown). These results indicate that both IL-1α and IL-1β are involved in LC migration, but not in LC maturation.

Effect of IL-1-deficiency on the induction of antigen-specific T cells

I have recently reported that IL-1 produced by APCs plays an important role in antigen-specific T cell priming [28]. Then, I examined the role of IL-1 in the induction of antigen-specific T cells by sensitizing with high dose of TNCB. LN T cells from IL-1-deficient mice sensitized with TNCB were cultured with TNP-conjugated and T cell-depleted wild-type splenocytes (Fig.3). Proliferative responses of IL-1$\alpha^{-/-}$ T cells as well as IL-1$\alpha/\beta^{-/-}$ T cells were markedly impaired, while those of IL-1$\beta^{-/-}$ T cells were comparable with those of wild-type T cells. Previously, I showed that T cell development and intrinsic T cell function is normal in IL-1-deficient mice [28]. Thus, the reduced proliferative responses of IL-1$\alpha^{-/-}$ and IL-1$\alpha/\beta^{-/-}$ T cells suggest that LN T cells from these IL-1-deficient mice were not sensitized sufficiently *in vivo*, because APCs were derived from wild-type mice.

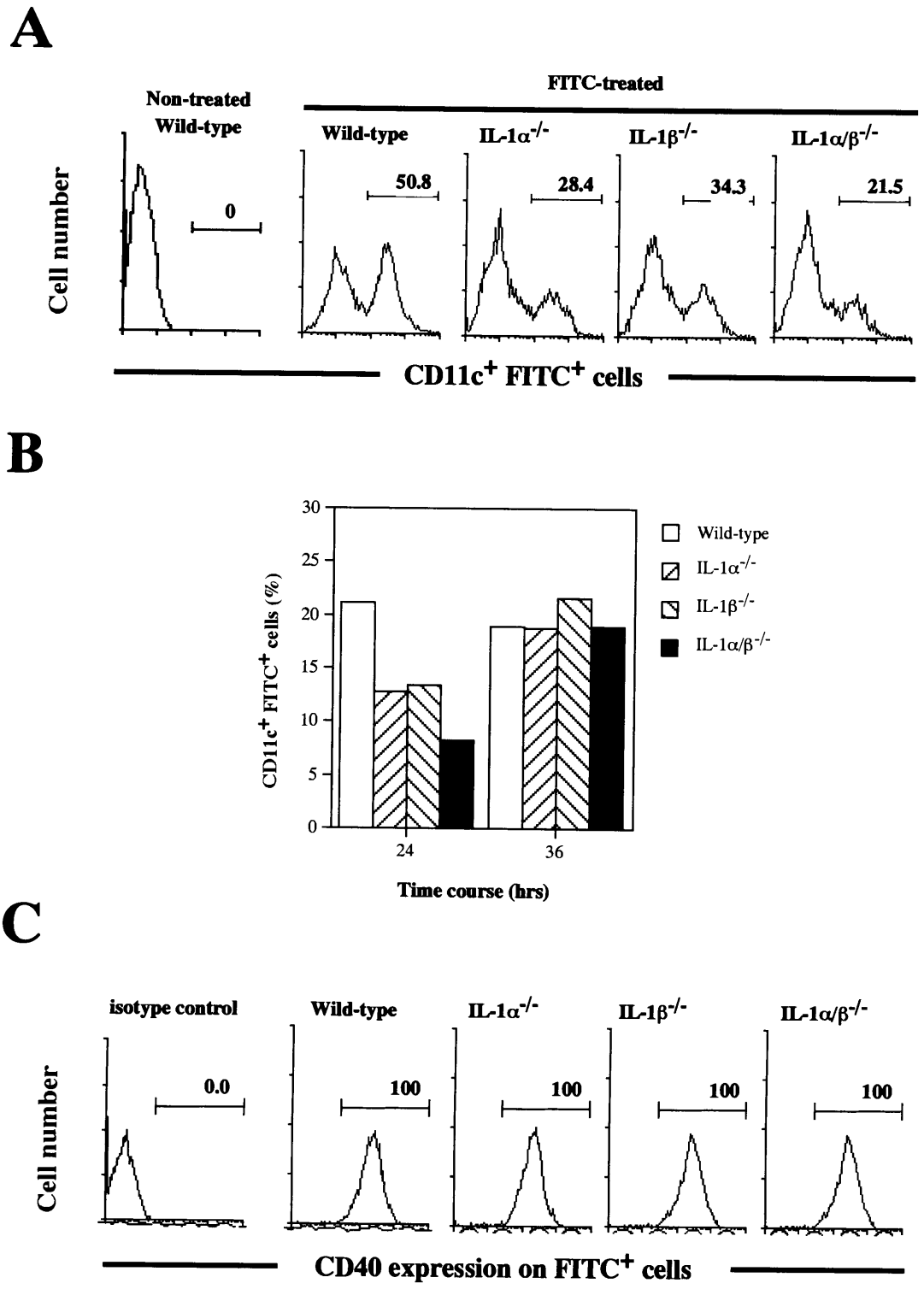

Figure 2. Effects of IL-1-deficiency on LC migration and maturation
Wild-type mice and IL-1-deficient mice were epicutaneously sensitized with 0.5% FITC. After FITC-painting, draining LNs were harvested and analyzed for FITC and CD40 expression by flow cytometry. (A) Content of FITC+ cells among CD11c+ cells at 24 h after FITC-painting. (B) Kinetics of CD11c+ FITC+ LC migration. (C) Expression of CD40 on CD11c+ FITC+ cells. One of representative data from six independent experiments is shown.

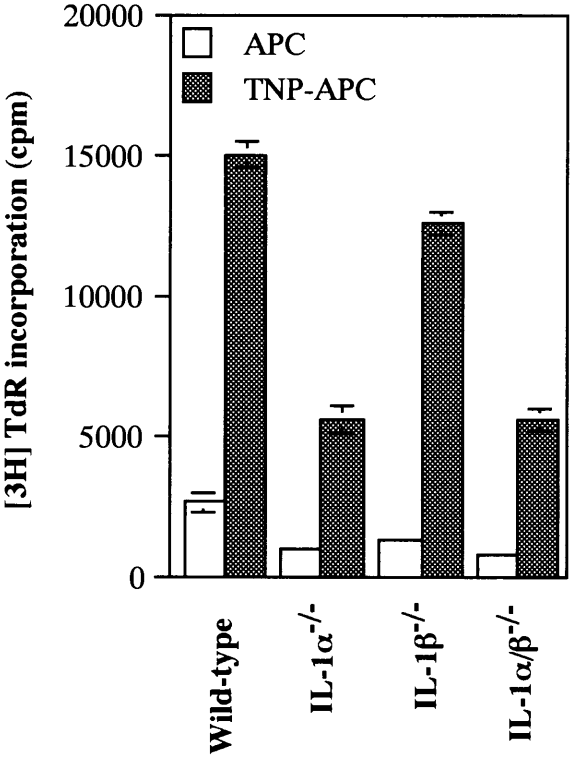

Figure 3. Proliferation of TNCB-sensitized LN T cells after *in vitro* restimulation
On day 5 after sensitization with 3.0% TNCB, LNs were harvested and T cells were purified through MACS columns. These T cells and irradiated TNP-conjugated APCs or non-treated APCs from wild-type splenocytes were cultured for 72 h, then the proliferative response was assessed by the incorporation of [3H]-thymidine. The data are reproducible in three-independent experiments.

IL-1α expression on LCs migrating into draining LNs

Although LCs are known as APCs in the sensitization phase of CHS, immature skin LCs do not produce IL-1α [32,33]. It is not known whether or not mature LCs which migrate from the skin into draining LNs produce IL-1α. I carried out flow cytometric analysis of the LN cells from FITC-painted wild-type mice after stimulation with LPS for 12 h. As shown in Fig. 4A and B, both $CD11c^+$ $FITC^+$ LN cells and $CD11b^+$ PECs produced IL-1α. Cells from IL-$1\alpha^{-/-}$ mice (Fig. 4A and B) and non-stimulated TGC-induced $CD11b^+$ PECs (data not shown) did not produce IL-1α. Agonistic anti-CD40 mAb-treated $CD11c^+FITC^+$ cells also produced IL-1α (data not shown).

When $CD4^+$ T cells from DO11.10 Tg mice were cultured with $CD11c^+$ cells from the skin or $CD11b^+$ PECs in the presence of the OVA peptide, $CD11c^+$ cells from the skin could activate DO.11.10 $CD4^+$ T cells approximately 5 times more strongly than TGC-induced $CD11b^+$ PECs (Fig. 4C), suggesting that LCs are the major APCs in this reaction. Under this condition, proliferative response of T cells cultured with $CD11c^+$ cells from IL-$1\alpha/\beta^{-/-}$ mice was low compared with that of wild-type mice, and was recovered to wild-type levels in the presence of rIL-1α (Fig. 4C). These results showed that mature LCs in draining LNs could produce IL-1α, which may play an important role in contact-allergen specific T cell activation.

Adoptive transfer of TNP-conjugated epidermal cells

As shown above, IL-1, especially IL-1α, may play an important role not only in LC migration but also in contact-allergen specific T cell activation. To examine roles of IL-1 produced by LCs in T cell activation, I performed adoptive transfer of TNP-conjugated wild-type or IL-1-deficient ECs, which contains approximately 2 % LCs, into wild-type mice which were not sensitized with contact-allergens. When TNP-conjugated IL-$1\alpha^{-/-}$ ECs as well as IL-$1\alpha/\beta^{-/-}$ ECs were transferred into wild-type mice, their ear swelling after treatment with 1.0 % TNCB was markedly reduced compared with these transferred with TNP-

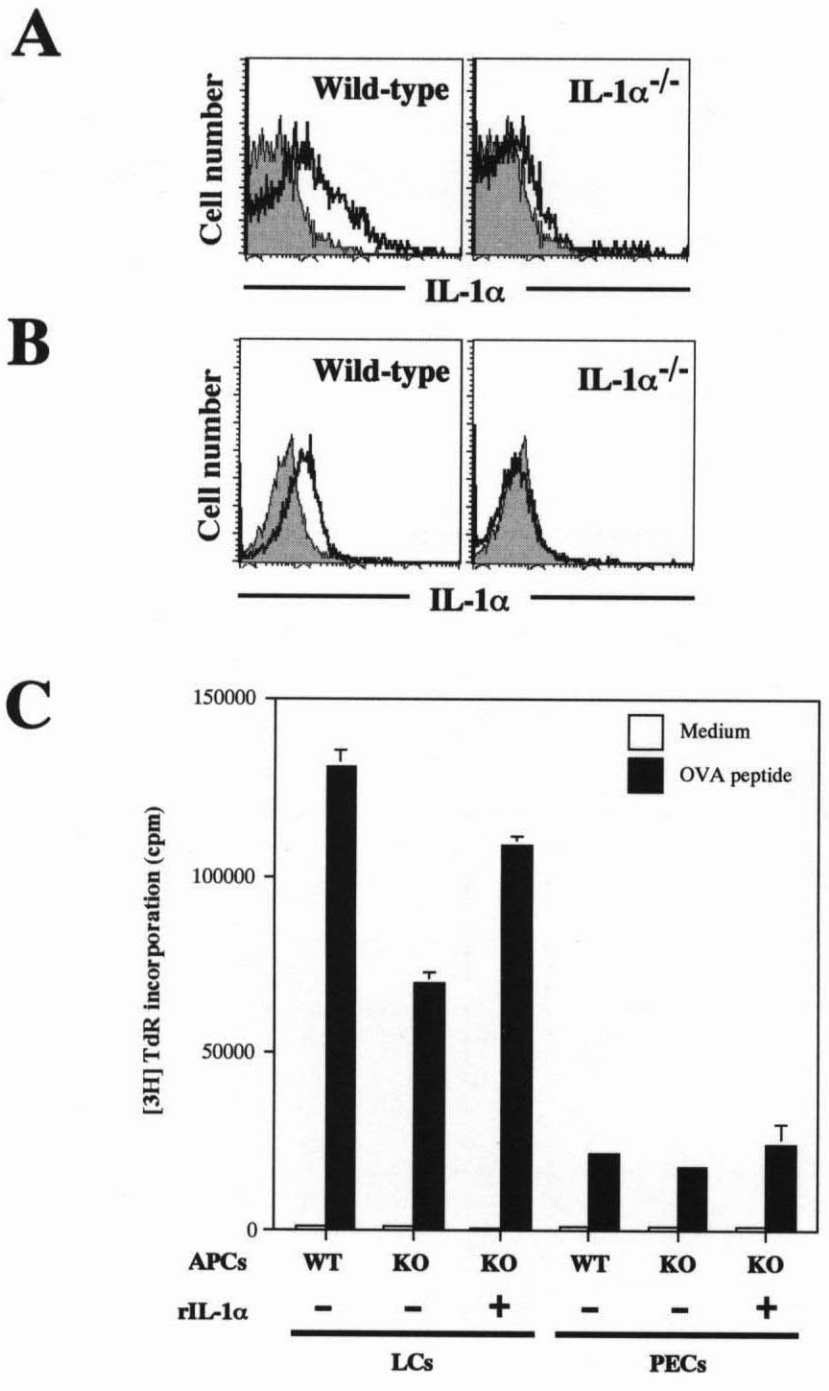

Figure 4. IL-1α expression in LCs migrated into draining LNs
Intracellular IL-1α in LPS-stimulated CD11b/Mac-1+ PECs (A) and in CD11c+ FITC+ LCs from LNs (B) was stained with PE-anti-mouse IL-1α mAb (solid lines) and with isotype-matched control IgG (shaded), and analyzed by flow-cytometry.
DO11.10 CD4+ T cells were cultured with either CD11c+ skin LCs or TGC-induced PECs from wild-type (WT) mice in the presence of the OVA peptide with or without rIL-1α for 72 h, then, the proliferative response was assessed by the incorporation of [3H]-thymidine (C). WT: wild-type mice, KO: IL-1α/β-/- mice.

conjugated wild-type ECs (Fig. 5). On the other hand, when transferred with TNP-conjugated IL-1$\beta^{-/-}$ ECs, ear swelling was similar to that of wild-type (Fig. 5). These results suggest that IL-1α, but not IL-1β, produced by APCs in ECs, most likely to be LCs, is required for TNP-specific T cell activation.

Distinctive roles of IL-1α and IL-1β in allergen-specific antibody production

To examine the possibility that IL-1 is involved in CHS through contact-allergen-specific antibody production, TNCB-specific antibody production was measured in those IL-1-deficient mice. TNCB-specific antibody production was measured in those IL-1-deficient mice. As shown in Fig. 6, serum TNP-specific IgG levels in IL-1$\beta^{-/-}$ mice as well as in IL-1$\alpha/\beta^{-/-}$ mice were lower than those in wild-type mice, while those in IL-1$\alpha^{-/-}$ mice were comparable. These results are consistent with our previous report [34] showing that IL-1β, but not IL-1α, is mainly involved in the antibody production. Thus, it was shown that molecular species of IL-1 is different between CHS and antibody production, suggesting different mechanisms are involved in those reactions.

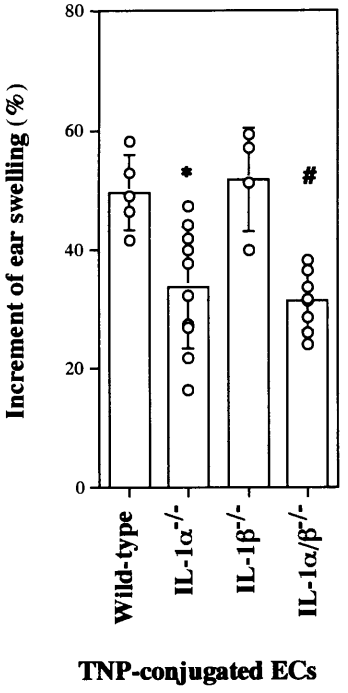

Figure 5. CHS induction in wild-type mice transferred with TNP-conjugated ECs from IL-1-deficient mice

TNP-conjugated ECs from wild-type or IL-1-deficient mice were injected into wild-type mice subcutaneously. After seven days, the ear was challenged with high dose TNCB, and ear swelling was measured 24 h later. Each circle represents an individual mouse, and an average and SD are shown. * $p < 0.005$ and # $p < 0.001$

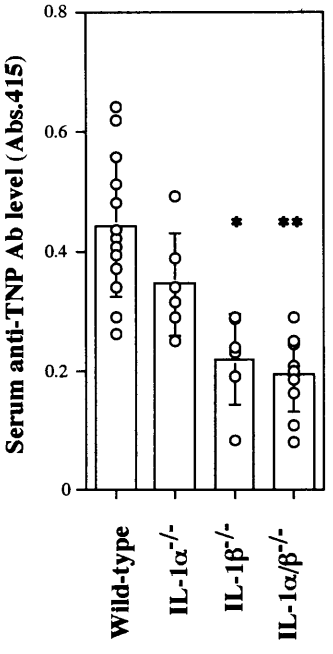

Figure 6. TNP-specific serum antibody levels in IL-1-deficient mice

TNP-specific immunoglobulin levels in sera were measured by ELISA. Results are expressed by the absorbancy at 415 nm. Each circle represents an individual mouse, and an average and SD are shown. Wild-type (n=13), IL-1α-/- (n=6), IL-1β-/- (n=6) and IL-1α/β-/- (n=10) mice. * $p < 0.005$, ** $p < 0.001$

Discussion

Previously, IL-1β rather than IL-1α was thought as a mediator of CHS, because CHS was suppressed by the administration of anti-IL-1β mAb, but not by anti-IL-1α mAb [10]. Furthermore, low dose TNCB-induced CHS was suppressed in IL-1β$^{-/-}$ mice, although high dose TNCB-induced CHS and Oxazolone-induced CHS were mounted normally in these mice [17,18]. Here, I demonstrated that TNCB-induced CHS was markedly impaired in IL-1α$^{-/-}$ mice as well as in IL-1α/β$^{-/-}$ mice, while that in IL-1β$^{-/-}$ mice was not significantly affected, indicating that IL-1α rather than IL-1β plays a crucial role in this CHS. Our results are consistent with the previous report using IL-1β$^{-/-}$ mice in which no effect was observed at high dose [18], but contrary to the results obtained by using IL-1 mAb [10]. However, not only high dose but also low dose TNCB-induced CHS was normal in IL-1β$^{-/-}$ mice in our system. I think that probably difference of the experimental conditions and genetic background of the mice may have affected the results [31]. In regard to this, I found that suppression of ear swelling in IL-1α$^{-/-}$ mice of the C57BL/6J background was similar to those of IL-1α/β$^{-/-}$ mice, whereas those of IL-1α$^{-/-}$ mice of the BALB/cA background was milder compared with those of IL-1α/β$^{-/-}$ mice (data not shown). Thus, the action of each IL-1 molecular species may be different in 129 x B6 background mice, that were used in the previous reports [17,18], from that in the C57BL/6J background mice that I used in this study.

LC migration was suppressed both in IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice. However, ear swelling was only suppressed in IL-1α$^{-/-}$ mice, indicating that the delayed LC migration may not completely explain the suppression of CHS in IL-1α$^{-/-}$ mice. On the other hand, I found that allergen-specific T cell priming was reduced in IL-1α$^{-/-}$ mice, but not in IL-1β$^{-/-}$ mice (Fig. 2 and Fig. 4C). Furthermore, I showed that ear swelling upon treatment with TNCB was significantly reduced in mice previously transferred with TNP-conjugated ECs from IL-1α$^{-/-}$ or IL-1α/β$^{-/-}$ mice compared with the mice transferred with those cells from wild-type mice or

IL-1β$^{-/-}$ mice. These observations suggest that IL-1α produced by APCs plays a crucial role in the initiation of primary immune response by activating allergen-specific T cells. In this context, I showed that IL-1α was produced by mature CD11c^{+} FITC^{+} LCs in LNs, although so far it was only believed that IL-1α was produced in skin KCs, but not in immature skin LCs [6-8]. Taken together, these observations suggest that IL-1α produced by mature LCs in LNs migrating from the skin plays an important role in allergen-specific T cell priming.

I demonstrated that IL-1α$^{-/-}$, but not IL-1β$^{-/-}$, produced by APCs was responsible for the proliferative responses of antigen-specific T cells. This observation suggests that IL-1α is required for the development of antigen-specific memory T cells. Although I tried to demonstrate that the proportion of memory T cells (CD44^{+} or CD45RB^{-} cells) in LN T cells was decreased in TNCB-sensitized IL-1α$^{-/-}$ mice compared to the wild-type mice, I could not detect the difference (data not shown). Probably, the proportion of TNP-specific memory T cells was too small to detect in total T cell population. At this moment, I cannot exclude the possibility completely that TNP-specific T cells of IL-1α$^{-/-}$ mice became anergy because of insufficient activation.

This makes clear contrast with the essential role of IL-1β in contact allergen-specific Ab production. Recently, I have also shown that Ab production against sheep red blood cells (SRBC) is severely impaired in IL-1β$^{-/-}$ mice, in agreement with present observation [34]. Thus, it is shown that IL-1α and IL-1β play distinct roles in CHS; IL-1α is mainly involved in contact-allergen specific T cell priming, while IL-1β is involved in contact-allergen-specific antibody production. In this context, it was reported that, in germinal centers of human tonsils, IL-1β, but not IL-1α, is strongly expressed in follicular dendritic cells (FDCs) which play important roles in affinity maturation and isotype switch of Igs through interaction with B cells [35]. Since Ig class switching depends on CD40-CD40L interaction, IL-1β may be required for the B cell-FDC interaction. Consistently with this notion, I have recently reported that IL-1 is required for the induction of CD40L on naive T cells [28]. On the other hand, it is

known that T cell priming through T cell-LC interaction occurs in the T cell zones in LNs. I showed that IL-1α plays a major role in this process. Thus, the functional specificity of IL-1α and IL-1β seems to depend on the difference of the producing cells and the sites of production.

It was reported that CHS was suppressed in $Fc\gamma R^{-/-}$ mice [36], suggesting antibodies were involved in this reaction. This observation apparently contradicts our data that hapten-specific antibody levels were not correlated with the development of CHS. However, since athymic mice displayed a similar neutrophil response in oxazolone induced contact dermatitis [36] and $MHCII^{-/-}$ mice in which $CD4^+$ cells are absent displayed elevated CHS [3], it seems unlikely that hapten-specific antibody is involved in this reaction. Similar dissociation between CHS sensitivity and allergen-specific antibody production was observed in $CD80/CD86^{-/-}$, $CD154^{-/-}$ and $OX40L^{-/-}$ mice [24,27,37]. However, it is not well resolved how FcγR is involved in CHS. With regard to this, Zhang and Tinkle have shown that production of inflammatory cytokines, such as TNFα and IL-1β, was reduced in $Fc\gamma R^{-/-}$ mice [36]. Since these cytokines are induced in the skin during the first allergen sensitization in mice due to the irritant effects of the chemicals, it is considered that allergen-specific Ig is not involved in this reaction. Therefore, FcγR may be involved in the activation of the cutaneous innate immune system that is important for the inflammatory cell infiltration and cytokine response [36]. It is possible that this defect of proinflammatory cytokine production is responsible for the diminished CHS in $Fc\gamma R^{-/-}$ mice.

In conclusion, I have shown that IL-1α, but not IL-1β, produced by mature LCs migrating into LNs is essential for contact-allergen specific T cell activation during the sensitization phase of CHS. Thus, IL-1α and IL-1β play clearly distinct roles in the immune system probably depending on the localization of the producing cells and responding cells in a lymphoid organ. I are now trying to further elucidate these complex regulatory mechanisms of the immune system involving IL-1α and IL-1β. These studies may provide important cues for the control of CHS.

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