**Table 3. In vitro cytokine production by splenocytes (5 × 10^{6} cells/mL) stimulated with heat-killed *Candida albicans* blastoconidia (10[7]/mL).**

| Strain | IFN-$\gamma$, pg/mL | IL-10, pg/mL |
|---|---|---|
| IL-1$\alpha^{+/+}\beta^{+/+}$ | 47.4 ± 30.7 | 32.3 ± 8.8 |
| IL-1$\alpha^{-/-}$ | <15.6[a] | 30.8 ± 12.2 |
| IL-1$\beta^{-/-}$ | <15.6[a] | <8[b] |
| IL-1$\alpha^{-/-}\beta^{-/-}$ | <15.6[a] | <8[b] |

**NOTE.** Data were obtained from 1 experiment and are expressed as the mean ± SD production in 5 mice/group. IFN, interferon; IL, interleukin.

[a] $P<.05$, vs. IL-1$\alpha^{+/+}\beta^{+/+}$ mice.

[b] $P<.05$, vs. IL-1$\alpha^{+/+}\beta^{+/+}$ and IL-1$\alpha^{-/-}$ mice.

granulocyte recruitment [17, 18], this effect most likely is instrumental in explaining the difference in recruitment between the IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice. However, it has to be taken into account that assessment of PMN recruitment to a peritoneal inflammatory stimulus is, at best, an approximation of early parenchymal PMN recruitment at the various sites of infection.

Transcription of the genes encoding MIP-2 and KC is induced through signals mediated by Toll-like receptor 4 [19], and IL-1 has been shown to selectively stabilize KC mRNA [20]. Hence, it is hypothesized that the normal production of MIP-2—in contrast to the 80% reduced production of KC by IL-1–deficient macrophages—is due to the selective effect of IL-1 on KC mRNA.

The effect of endogenous IL-1 on PMN function has not been investigated previously. The results of the present study indicate that IL-1 affects PMN function and that the modes of action differ for IL-1$\alpha$ and IL-1$\beta$. Whereas IL-1$\beta^{-/-}$ PMNs showed impaired superoxide production, IL-1$\alpha^{-/-}$ PMNs showed a decreased capacity to damage *C. albicans* pseudohyphae, indicating that IL-1$\alpha$ is important for PMN degranulation. In addition, production of IL-6, which is induced by IL-1 and known to stimulate PMN function [21–23], was reduced only in IL-1$\beta^{-/-}$ mice, and this may further contribute to impaired PMN function and reduced anticandidal defense. Since the *C. albicans* burden in IL-1$\alpha^{-/-}$ kidneys was significantly higher than that in IL-1$\beta^{-/-}$ kidneys on day 7 of infection, it is suggested that the initially reduced PMN recruitment in IL-1$\beta^{-/-}$ mice is overridden by the effect of the reduced capacity to kill *C. albicans* pseudohyphae in IL-1$\alpha^{-/-}$ mice. However, despite the difference in outgrowth between the 2 mouse strains on day 7, IL-1$\beta^{-/-}$ mice showed susceptibility to infection similar to that of IL-1$\alpha^{-/-}$ mice. Most of the apparent discrepancy is explained by the fact that, on day 14 of infection, the fungal burden in both IL-1$\alpha^{-/-}$ mice and IL-1$\beta^{-/-}$ mice was equally increased. Another possible explanation for this observation is that, in addition to fungal outgrowth, the additional inflammatory damage in the organs of IL-1$\beta^{-/-}$ mice may also have contributed to mortality.

If the beneficial effect of endogenous IL-1 is exerted only through PMNs, the differences in outgrowth between the groups, as observed in nongranulocytopenic mice, should disappear in granulocytopenic mice. However, the observed differences persisted, indicating that the effect of endogenous IL-1 is at least partly mediated through cells or mechanisms other than modulation of PMN recruitment and function, which is in line with previous findings [3, 4]. Nevertheless, because cyclophosphamide also reduces lymphocyte numbers, it cannot be excluded that a minor beneficial effect of IL-1 on *C. albicans* outgrowth may be mediated through PMNs.

Macrophages and lymphocytes are other cells that are likely to mediate the protective effect of IL-1 [24, 25]. Most importantly, IFN-$\gamma$ production was found to be absent in IL-1–deficient splenocytes. This is in agreement with previous data showing that endogenous IL-1 is important for the production IFN-$\gamma$ during *C. albicans* stimulation of whole blood [26]. The crucial role of IFN-$\gamma$ in host defense has become apparent from studies showing that IFN-$\gamma$–deficient mice are highly susceptible to disseminated candidiasis and that administration of recombinant IFN-$\gamma$ reduces outgrowth [12, 27, 28]. Recently, we observed that delayed development of a type 1 response—that is, delayed IFN-$\gamma$ production—contributed to increased outgrowth in localized *C. albicans* infection [9]. Furthermore, we observed foreign body giant cells (FBGCs) in the kidneys of IL-1–deficient mice. The transition of macrophages to functionally inactive FBGCs is favored in the presence of type 2 cytokines [29]. Therefore, the presence of FBGCs points to an anti-inflammatory type 2 response, which has been shown to be detrimental to anticandidal host defense [30].

In conclusion, the present study has further clarified the mechanisms through which IL-1$\alpha$ and IL-1$\beta$ enhance host resistance against candidiasis. Interestingly, the absence of either IL-1$\alpha$ or IL-1$\beta$ had divergent consequences. Although they are products of different genes, IL-1$\alpha$ and IL-1$\beta$ are highly homologous and have similar tridimensional structures and common receptors. It is unclear at this point what determines the functional differences in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice—a different distribution of cytokines in the organs, the known dichotomy between cell-bound IL-1$\alpha$ and secreted IL-1$\beta$ [31], or differential receptor affinity. Although a functional difference between IL-1$\alpha$ and IL-1$\beta$ was observed in this study, both IL-1$\alpha$ and IL-1$\beta$ were shown to be essential for anticandidal host defense. The complementary roles of IL-1$\alpha$ and IL-1$\beta$ in host defense against *C. albicans* infection are further underscored by the even higher susceptibility to disseminated candidiasis of mice lacking both IL-1$\alpha$ and IL-1$\beta$. These data constitute an additional body of evidence that IL-1–dependent mechanisms play an important role in innate host defense against *C. albicans.*

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# *Original Article*

# Combined Interleukin-6 and Interleukin-1 Deficiency Causes Obesity in Young Mice

**Dai Chida,[1] Toshimasa Osaka,[2] Okito Hashimoto,[1] and Yoichiro Iwakura[1]**

**Proinflammatory cytokines including interleukin (IL)-1 and IL-6 exert pleiotropic effects on the neuro-immuno-endocrine system. Previously, we showed that IL-1 receptor antagonist–deficient (IL-1Ra$^{-/-}$) mice show a lean phenotype due to an abnormal lipid metabolism. On the contrary, it was reported that IL-6$^{-/-}$ mice exhibit obesity after 6 months of age. This study sought to assess the roles of IL-1 and IL-6 in body weight homeostasis. We generated mice deficient in IL-6 and IL-1Ra (IL-6$^{-/-}$ IL-1Ra$^{-/-}$) and IL-6, IL-1α, and IL-1β (IL-6$^{-/-}$ IL-1$^{-/-}$). IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice exhibited a lean phenotype, similar to IL-1Ra$^{-/-}$ mice. On the other hand, IL-6$^{-/-}$ IL-1$^{-/-}$ mice became obese as early as 10 weeks of age, while IL-1$^{-/-}$ mice and IL-6$^{-/-}$ mice were normal at this age. The daily food intake was significantly higher in IL-6$^{-/-}$ IL-1$^{-/-}$ mice than in IL-6$^{-/-}$ IL-1$^{+/-}$ mice, while energy expenditure was comparable in these two strains. Acute anorexia induced by peripheral administration of IL-1 was significantly suppressed in IL-6$^{-/-}$ IL-1$^{-/-}$ mice, but not in IL-1$^{-/-}$ mice or IL-6$^{-/-}$ mice compared with wild-type mice. These results indicate that IL-1 and IL-6 are both involved in the regulation of body fat in a redundant manner in young mice. *Diabetes* 55:971–977, 2006**

Interleukin (IL)-1, a major mediator of inflammation, also performs numerous functions related to host defense mechanisms by regulating not only the immune system but also the neural and endocrine systems. IL-1 is produced by a wide variety of cells, including monocytes, macrophages, epithelial cells, endothelial cells, and glial cells. IL-1 receptors are expressed on a wide range of immune, neural, and endocrine cells, reflecting the pleiotropic activities of this molecule (1). Endogenous IL-1 in the brain plays a pivotal role in hypothalamic cytokine expression and the development of anorexia (2). Leptin, released from adipocytes, exerts an inhibitory feedback effect on fat masses by acting on hypothalamic nuclei that express the cognate signal-transducing receptor, ObRb (3). IL-1, which is induced by leptin, is involved in the leptin-induced suppression of feeding (4). It is interesting to note that serum IL-1 receptor antagonist (IL-1Ra) levels are sevenfold higher in human patients with obesity in comparison with nonobese subjects (5). In addition, a large quantity of IL-1Ra is secreted from adipose tissue, although the biological significance of this phenomenon is not well understood (6). Using IL-1$^{-/-}$ and IL-1Ra$^{-/-}$ mice, we demonstrated the physiological role of IL-1 in feeding behavior and energy metabolism. IL-1Ra$^{-/-}$ mice have a defect in lipid accumulation in adipose tissue, exhibiting leanness (7). Recently, Garcia et al. (8) demonstrated that IL-1R$^{-/-}$ mice developed mature-onset obesity, beginning to deviate from the weight of wild-type mice at 5–6 months of age.

The acute-phase immunoregulatory cytokine IL-6 is secreted from adipose tissue during noninflammatory conditions in humans. Serum IL-6 levels correlate with BMI (9–11), as seen for leptin. Unlike leptin, however, IL-6 is coexpressed with its receptor by neurons of the hypothalamic nuclei that regulate body composition (12,13) and nonneuronal cells, such as astrocytes, microglia, and brain endothelial cells (14). Previous studies have demonstrated that IL-6$^{-/-}$ mice develop mature-onset obesity; IL-6 exerts antiobesity effects centrally by increasing energy expenditure (15,16). Furthermore, the respiratory exchange ratio was higher in young IL-6$^{-/-}$ mice than in wild-type mice, indicating that these mutant animals preferentially oxidize carbohydrates over fat (17).

The fact that IL-6, which is strongly induced by IL-1, shares many biological functions with IL-1 prompted us to investigate the relationship between IL-1 and IL-6 and their roles in body weight homeostasis. To address this issue, we created double knockout mice deficient in both IL-1Ra and IL-6 and triple knockout mice lacking IL-1α, IL-1β, and IL-6. We found that IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice are as lean as IL-1Ra$^{-/-}$ mice. In contrast, IL-6$^{-/-}$ IL-1$^{-/-}$ mice become obese earlier than IL-6$^{-/-}$ mice, beginning to deviate from IL-6$^{-/-}$ mice at 10 weeks of age. These results demonstrate that the effect of IL-1 on body weight homeostasis is independent of IL-6 and that deficiency of IL-1 and IL-6 synergistically induce obesity. We also found that IL-6$^{-/-}$ IL-1$^{-/-}$ mice are hyperphagic in comparison with IL-6$^{-/-}$ animals, despite similar energy expenditures.

## RESEARCH DESIGN AND METHODS

**Reagents.** Recombinant murine IL-1α was obtained from Pepro Tech EC (London, U.K.). The lyophilized protein was dissolved in pyrogen-free 0.9% NaCl (saline) containing 0.1% BSA (Sigma, A9306).

IL-1Ra$^{-/-}$ mice and mice deficient for both IL-1α and IL-1β (IL-1$^{-/-}$ mice) were produced as described (18). IL-6$^{-/-}$ mice were kindly provided by Dr. Manfred Kopf (19). These mice were backcrossed to C57BL/6J mice for eight generations. IL-6$^{-/-}$ IL-1$^{-/-}$ mice were produced by crossing the IL-1$^{-/-}$ and IL-6$^{-/-}$ mice, while IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice were produced by crossing

From the [1]Division of Cell Biology, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan; and the [2]National Institute of Health and Nutrition, Shinjuku, Japan.

Address correspondence and reprint requests to Yoichiro Iwakura, DSc, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Toyko 108-8639, Japan. E-mail: iwakura@ims.u-tokyo.ac.jp.

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eWAT, epididymal white adipose tissue; FFA, free fatty acid; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; NPY, neuropeptide Y; TAG, triacylglycerol.

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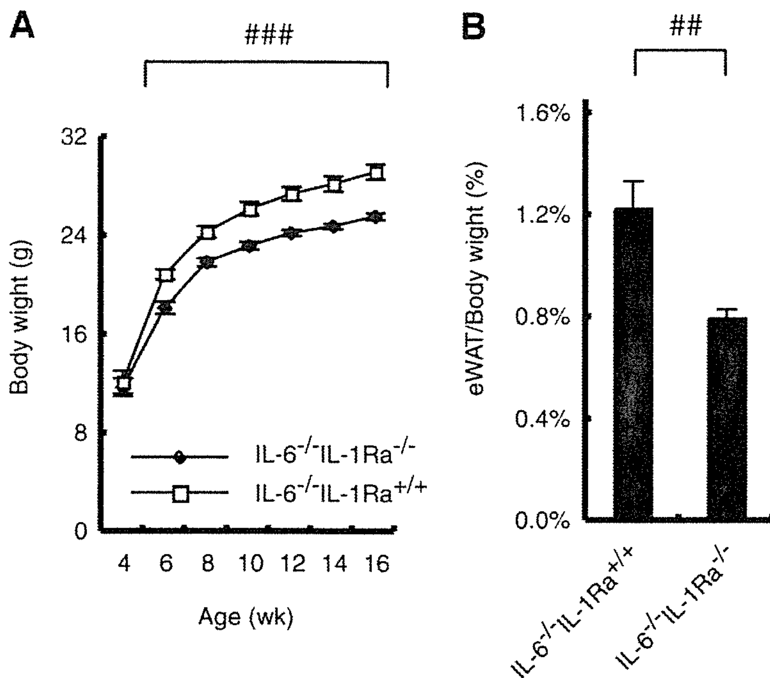


**FIG. 1. Growth curves and eWAT volume in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ male mice.** ***A*****: Growth curves of IL-6$^{-/-}$ IL-1Ra$^{-/-}$ ($n$ = 8) (♦) and IL-6$^{-/-}$ IL-1Ra$^{+/+}$ ($n$ = 15) (□) mice fed by normal diet ad libitum.** ***B*****: eWAT volume in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ and IL-6$^{-/-}$ IL-1Ra$^{+/+}$ mice at 16 weeks. Data are expressed as means ± SE. Statistical significance was calculated by repeated-measures ANOVA and Student's $t$ test (*A*) or Student's $t$ test (*B*). ##$P$ < 0.01; ###$P$ < 0.001.**

IL-1Ra$^{-/-}$ and IL-6$^{-/-}$ mice. For each experiment, IL-6$^{-/-}$ IL-1$^{-/-}$ mice were produced by crossing IL-6$^{-/-}$ IL-1$^{-/-}$ male mice with IL-6$^{-/-}$ IL-1$^{+/-}$ female mice. IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice were generated by crossing IL-6$^{-/-}$ IL-1Ra$^{+/-}$ male mice with IL-6$^{-/-}$ IL-1Ra$^{+/-}$ female mice. After weaning, mice were housed individually at 4 weeks of age. Age-matched male littermates or adult (9–16 weeks of age) male mice obtained from a breeder (SLC, Shizuoka, Japan) were used for each experiment. When necessary, mice produced by in vitro fertilization were used to obtain large numbers of mice at one time under the same conditions. Single knockout mice were obtained from homozygous crossings. Mice were kept under specific pathogen-free conditions in environmentally controlled clean rooms at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. Animals were housed at an ambient temperature of 24°C under a daily 12-h light (800–2000) and 12-h darkness cycle. All experiments were performed according to the institutional ethical guidelines for animal experimentation and according to safety guidelines for gene manipulation experiments.

**Body weight and food intake measurement.** Body weight and food intake of IL-6$^{-/-}$ IL-1Ra$^{+/+}$ and IL-6$^{-/-}$ IL-1Ra$^{-/-}$ pups, derived from the crosses of IL-6$^{-/-}$ IL-1Ra$^{+/-}$ males and IL-6$^{-/-}$ IL-1Ra$^{+/-}$ females, were measured once a week in the afternoon, beginning from the day of weaning at 4 weeks of age. Body weight and food intake of IL-6$^{-/-}$ IL-1$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{+/-}$ pups, derived from the crosses of IL-6$^{-/-}$ IL-1$^{-/-}$ males with IL-6$^{-/-}$ IL-1$^{+/-}$ females, were measured once a week in the afternoon, beginning from the day of weaning at 4 weeks of age. The obese phenotype in IL-6$^{-/-}$ IL-1$^{-/-}$ mice was independently confirmed in mice derived from in vitro fertilization using IL-6$^{-/-}$ IL-1$^{-/-}$ sperm with IL-6$^{-/-}$ IL-1$^{-/-}$, $^{+/-}$, or $^{+/+}$ eggs. Epididymal white adipose tissue (eWAT) and the soleus muscle were dissected and weighed at the indicated times. Male mice at 16 weeks of age were subjected to food restriction for 5 days by administration of 5% of their body weight of normal diet. Male mice at 12–14 weeks of age were subjected to IL-1 treatment. Mice were housed individually 1 week before the experiment, and peripheral IL-1 (1 μg/kg body wt i.p.) injection was performed at 1700. Food intake was measured 24 h after injection.

**Measurement of body temperature.** The intraperitoneal body temperatures of mice were measured using a telemetry system (ELAMS system; BioMedic Data System, Maywood, NJ), as described previously (20). Mice were moved to a cold room (4°C) at 1000. Body temperature was then recorded at regular intervals over the next 24 h.

**Indirect calorimetry.** Whole-body $O_2$ consumption and $CO_2$ production were measured in a respiration chamber, measuring 140 × 80 × 90 mm in size, ventilated with fresh air at a rate of 200 ml/min. The difference in concentrations of $O_2$ and $CO_2$ between inflow and outflow air was measured with a differential $O_2$ analyzer (LC700E; Toray, Tokyo, Japan) and two $CO_2$ sensors (GMW22D; Vaisala, Helsinki, Finland), respectively. Each mouse was placed in the chamber for 23 h. To avoid the influence of emotional thermogenic responses to cage-exchange stress, the data recorded during the 1st h were not analyzed. The results were then corrected for metabolic body mass ($g^{0.75}$).

**Blood constituents.** To analyze blood constituents, 16-week-old IL-6$^{-/-}$ IL-1Ra$^{-/-}$, IL-6$^{-/-}$, IL-1Ra$^{-/-}$, and wild-type mice and 13-week-old IL-6$^{-/-}$ IL-1$^{-/-}$, IL-6$^{-/-}$, IL-1$^{-/-}$, and wild-type mice were examined. Blood glucose levels were measured by the glucose oxidase method (Terumo), while serum triacylglycerol (TAG) and free fatty acid (FFA) levels were examined by colorimetric assays (triglyceride-E and NEFA-C tests, respectively; Wako Pure Chemical Industries). Serum insulin and leptin levels were both measured by enzyme-linked immunosorbent assay (Seikagaku, Tokyo, Japan) and radioimmunoassay (Eiken, Tokyo, Japan).

**Statistical analysis.** All values were calculated as means ± SE. Differences among body weight curves, food intake, and body temperatures were evaluated by a repeated-measures ANOVA, in which factor 1 was the between-groups factor and factor 2 was the within-subject factor (the different ages). Comparisons of the two groups were analyzed by the Student's $t$ test. To compare more than two groups, ANOVA was performed followed by Sheffe's tests. In all analyses, a two-tailed probability of <5% (i.e., $P$ < 0.05) was considered to be statistically significant.

## RESULTS

**IL-1Ra$^{-/-}$ mice are lean, independent of IL-6 action.** If excess IL-1 signaling in the IL-1Ra$^{-/-}$ mice causes leanness solely by enhancing IL-6 signaling, then IL-6 deficiency should cancel the lean phenotype seen in IL-1Ra$^{-/-}$ mice. By 6 weeks of age, IL-6$^{-/-}$ IL-1Ra$^{-/-}$ male mice exhibited leanness in comparison to IL-6$^{-/-}$ male mice (18.3 ± 0.5 g IL-6$^{-/-}$ IL-1Ra$^{-/-}$ [$n$ = 8] vs. 20.9 ± 0.4 g IL-6$^{-/-}$ IL-1Ra$^{+/+}$ [$n$ = 15], $P$ < 0.01) (Fig. 1*A*). eWAT weight per body weight at 16 weeks of age was significantly reduced in male IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice in comparison to male IL-6$^{-/-}$ (Fig. 1*B*; Table 1), while skeletal muscle weight was not significantly different (data not shown). These results demonstrate that excess IL-1 signaling promotes leanness independent of IL-6 signaling.

**IL-1$^{-/-}$ mice exhibit normal weight gain until 8 months.** Considering the fact that IL-1Ra$^{-/-}$ mice show leanness (7) and IL-1R$^{-/-}$ mice develop obesity (8), we asked whether IL-1$^{-/-}$ mice develop obesity. To exclude the effect of maternal effect, we analyzed pups delivered from ICR mice transferred with eggs made by in vitro fertilization. IL-1$^{-/-}$ and IL-1$^{+/-}$ pups were analyzed at 8 months. As demonstrated in Table 2, they were not significantly different in body weight, eWAT volume, glucose,

TABLE 1
Body weight, eWAT weight, and serum parameters in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice at 16 weeks of age

| | Body weight (g) | WAT (g) | WAT/body weight (%) | Insulin (pg/ml) | Leptin (pg/ml) | Nonesterified fatty acids (mEq/l) |
|---|---|---|---|---|---|---|
| Wild type | 27.2 ± 0.2 | 0.30 ± 0.01 | 1.10 ± 0.03 | 1,419 ± 40 | 919 ± 26 | ND |
| IL-1Ra$^{-/-}$ | 24.3 ± 0.3* | 0.21 ± 0.01* | 0.87 ± 0.02* | 972 ± 31† | 636 ± 38† | ND |
| IL-6$^{-/-}$ | 27.9 ± 0.1 | 0.34 ± 0.01 | 1.22 ± 0.03 | 1,631 ± 59 | 859 ± 28 | 1.19 ± 0.02 |
| IL-6$^{-/-}$ IL-1Ra$^{-/-}$ | 24.5 ± 0.2* | 0.21 ± 0.01* | 0.85 ± 0.02* | 931 ± 70† | 533 ± 33† | 1.11 ± 0.04 |

Data are means ± SE. Statistical significance was calculated by ANOVA followed by either Scheffe's test (body weight, WAT, WAT/body weight, insulin, and leptin) or the Student's $t$ test (nonesterified fatty acid). *$P < 0.01$; †$P < 0.05$. ND, not determined.

and insulin level. These results indicate that IL-1$^{-/-}$ mice show normal body weight homeostasis under our experimental conditions until 8 months.

**Deficiency of IL-1 and IL-6 synergistically induces obesity.** To examine the possible compensatory effect of IL-6 signaling on body weight homeostasis in IL-1$^{-/-}$ mice, we next analyzed the effect of IL-1 deficiencies on IL-6$^{-/-}$ mice. By 10 weeks of age, a significant increase in body weight was evident in the male IL-6$^{-/-}$ IL-1$^{-/-}$ mice in comparison to IL-6$^{-/-}$ IL-1$^{+/-}$ mice (Fig. 2*A*). At 13 weeks, body weight, eWAT weight, and eWAT weight/body weight in male IL-6$^{-/-}$ IL-1$^{-/-}$ mice increased significantly in comparison to IL-6$^{-/-}$ IL-1$^{+/-}$ animals (Fig. 2*B–D*). Again, skeletal muscle weight was not significantly different (data not shown). Body weight and eWAT volume of IL-1$^{-/-}$ and IL-6$^{-/-}$ mice were not significantly different from wild-type mice at 13 weeks (Fig. 2*B–D*), consistent with the previous reports demonstrating that IL-6$^{-/-}$ mice develop mature-onset obesity (15) and that IL-1$^{-/-}$ mice exhibit normal weight gain until 20 weeks (7). These results indicate that there is mutual redundancy for the suppression of body fat gain by IL-6 and IL-1 in young mice. The lean and obese phenotypes seen in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{-/-}$ mice, respectively, were only obvious in male mice, as previously seen with IL-1Ra$^{-/-}$ female mice, whose body weight was indistinguishable from wild-type mice (7).

We measured basal levels of glucose, insulin, leptin, TAG, and FFAs in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{-/-}$ mice. Serum insulin and leptin levels in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ male mice were significantly lower than those measured in IL-6$^{-/-}$ IL-1Ra$^{+/+}$ mice under fasting conditions, while glucose, TAG, and FFA levels were not significantly different (Table 1; data not shown). IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice did not differ significantly from IL-1Ra$^{-/-}$ mice in any of the parameters we examined (Table 1). Serum leptin levels in IL-6$^{-/-}$ IL-1$^{-/-}$ mice, however, were significantly higher than those observed in IL-6$^{-/-}$ IL-1$^{+/-}$ mice under fasting conditions (Fig. 2*F*), despite comparable levels of other serum constituents, including glucose (153 ± 9 mg/dl IL-6$^{-/-}$ IL-1$^{-/-}$ vs. 150 ± 10 mg/dl IL-6$^{-/-}$ IL-1$^{+/-}$; NS), insulin (Fig. 2*E*), TAG (138 ± 14 mg/dl IL-6$^{-/-}$ IL-1$^{-/-}$ vs. 175 ± 14 mg/dl IL-6$^{-/-}$ IL-1$^{+/-}$; NS), and FFAs (1.14 ± 0.05 mEq/l IL-6$^{-/-}$ IL-1$^{-/-}$ vs. 1.14 ± 0.06 mEq/l IL-6$^{-/-}$ IL-1$^{+/-}$; NS), between IL-6$^{-/-}$ IL-1$^{-/-}$ mice and IL-6$^{-/-}$ IL-1$^{+/-}$ mice.

**Increase in food intake in IL-6$^{-/-}$ IL-1$^{-/-}$ mice.** As obesity develops when energy intake (feeding) chronically exceeds total body energy expenditure (21), we measured food intake (Fig. 3*A* and *B*) and energy expenditure in IL-6$^{-/-}$ IL-1$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{+/-}$ mice (Fig. 3*C*). IL-6$^{-/-}$ IL-1$^{-/-}$ mice exhibited modest hyperphagia, beginning at 8 weeks of age, a time at which body weight did not differ significantly from IL-6$^{-/-}$ IL-1$^{+/-}$ mice (Fig. 3*A*). Both total food intake over 7–10 weeks and total food intake per body weight at 7 weeks were significantly increased in IL-6$^{-/-}$ IL-1$^{-/-}$ mice in comparison to IL-6$^{-/-}$ IL-1$^{+/-}$ mice (Fig. 3*B*). Food intake in IL-6$^{-/-}$ IL-1$^{-/-}$ mice was also significantly increased from the quantities observed for IL-6$^{-/-}$ IL-1$^{+/-}$ mice after 10 weeks, and food intake/body weight in IL-6$^{-/-}$ IL-1$^{-/-}$ mice older than 10 weeks was also higher than the controls, although it was not significant (data not shown). These results indicate that hyperphagia contributes to increase in food intake and obesity in IL-6$^{-/-}$ IL-1$^{-/-}$ mice.

We then examined the possibility that energy expenditure may be decreased in IL-6$^{-/-}$ IL-1$^{-/-}$ mice in comparison with control mice. We first analyzed the rate of weight loss following food restriction in IL-6$^{-/-}$ IL-1$^{-/-}$ mice. At 16 weeks of age, a point at which obesity was obvious in IL-6$^{-/-}$ IL-1$^{-/-}$ mice, animals were fed with a 5% body weight standard laboratory diet. Weight reduction over 5 days in all animals was not significantly different ($P$ = 0.330, $F$ = 4.600) (data not shown). We then assessed possible effects on cold sensitivity in IL-6$^{-/-}$ IL-1$^{-/-}$ mice. After exposure to 4°C, the rectal temperatures of IL-6$^{-/-}$ IL-1$^{-/-}$ mice were not significantly different from those observed in wild-type mice ($P$ = 0.783, $F$ = 5.591) (data not shown), suggesting that adaptive thermogenesis in IL-6$^{-/-}$ IL-1$^{-/-}$ mice did not differ significantly. Finally, using indirect calorimetry, we measured the metabolic rates of IL-6$^{-/-}$ IL-1$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{+/-}$ mice at 8–10 weeks of age, a time at which the body weights of these animals were not significantly different. Oxygen consumption in IL-6$^{-/-}$ IL-1$^{-/-}$ mice show a tendency to decrease in comparison to IL-6$^{-/-}$ IL-1$^{+/-}$ mice (Fig. 3*C*). The 24-h

TABLE 2
Body weight, eWAT weight, and serum parameters in IL-1$^{-/-}$ mice at 8 months of age

| | Body weight (g) | WAT (g) | WAT/body weight (%) | Glucose (mg/dl) | Insulin (pg/ml) | Nonesterified fatty acids (mEq/l) |
|---|---|---|---|---|---|---|
| IL-1$^{+/-}$ | 38.8 ± 3.2 | 1.23 ± 0.17 | 3.14 ± 0.30 | 168 ± 12 | 2,140 ± 500 | 1.11 ± 0.07 |
| IL-1$^{-/-}$ | 39.7 ± 2.7 | 1.65 ± 0.36 | 4.06 ± 0.74 | 149 ± 5 | 3,010 ± 670 | 0.94 ± 0.10 |

Data are means ± SE. Statistical significance was calculated by Student's $t$ test.

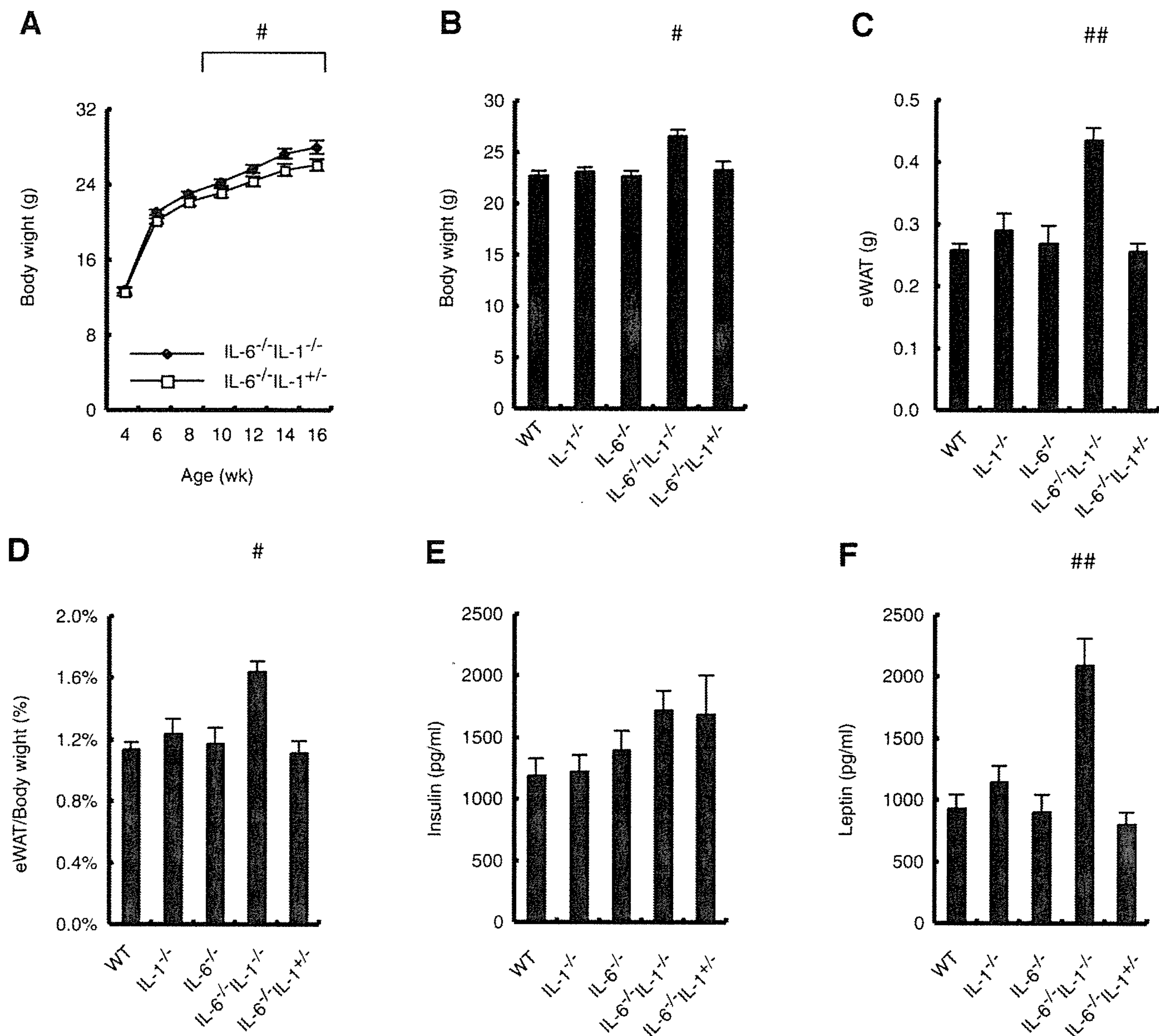


FIG. 2. Growth curves, eWAT volume, serum insulin, and leptin levels in IL-$6^{-/-}$ IL-$1^{-/-}$ male mice. *A*: Growth curves of IL-$6^{-/-}$ IL-$1^{-/-}$ ($n$ = 14) (♦) and IL-$6^{-/-}$ IL-$1^{+/-}$ ($n$ = 12) (□) mice, fed by normal diet ad libitum. *B*: Body weight in wild-type (WT), IL-$1^{-/-}$, IL-$6^{-/-}$, IL-$6^{-/-}$ IL-$1^{-/-}$, and IL-$6^{-/-}$ IL-$1^{+/-}$ mice at 13 weeks. *C*: eWAT volume in wild-type (WT), IL-$1^{-/-}$, IL-$6^{-/-}$, IL-$6^{-/-}$ IL-$1^{-/-}$, and IL-$6^{-/-}$ IL-$1^{+/-}$ mice at 13 weeks. *D*: eWAT volume/body weight in wild-type (WT), IL-$1^{-/-}$, IL-$6^{-/-}$, IL-$6^{-/-}$ IL-$1^{-/-}$, and IL-$6^{-/-}$ IL-$1^{+/-}$ mice at 13 weeks. *E*: Serum insulin levels in wild-type (WT), IL-$1^{-/-}$, IL-$6^{-/-}$, IL-$6^{-/-}$ IL-$1^{-/-}$, and IL-$6^{-/-}$ IL-$1^{+/-}$ mice at 13 weeks. *F*: Serum leptin levels in wild-type (WT), IL-$1^{-/-}$, IL-$6^{-/-}$, IL-$6^{-/-}$ IL-$1^{-/-}$, and IL-$6^{-/-}$ IL-$1^{+/-}$ mice at 13 weeks. Data are expressed as means ± SE. Statistical significance was calculated by repeated-measures ANOVA and Student's $t$ test (*A*) or by one-way ANOVA followed by Scheffe's test (*B–F*). #$P$ < 0.05; ##$P$ < 0.01.

average respiratory exchange ratio also did not differ significantly (IL-$6^{-/-}$ IL-$1^{-/-}$ mice: 0.87 ± 0.01; IL-$6^{-/-}$ IL-$1^{+/-}$ mice: 0.87 ± 0.01). Collectively, we failed to reveal any significant differences in energy expenditure in IL-$6^{-/-}$ IL-$1^{-/-}$ mice.

**Food intake after IL-1α treatment is higher in IL-$6^{-/-}$ IL-$1^{-/-}$ mice.** IL-1 plays a central role in central nervous system–mediated host defense mechanisms, including fever development, anorexia, and body weight loss (22–24). As previous studies demonstrated that IL-$6^{-/-}$ mice are resistant to IL-1–induced fever development (20,25), we examined the possibility that IL-$6^{-/-}$ mice are resistant to IL-1–induced anorexia (Fig. 4). Anorexia was assessed in IL-$6^{-/-}$ IL-$1^{-/-}$, IL-$1^{-/-}$, IL-$6^{-/-}$, and wild-type mice following a peripheral injection of mouse IL-1α. IL-$6^{-/-}$ IL-$1^{-/-}$ mice were resistant to IL-1α–induced reduction in food intake/body weight in comparison with IL-$1^{-/-}$ and wild-type mice. Food intake/body weight after IL-1α treatment was higher in IL-$6^{-/-}$ IL-$1^{-/-}$ mice than in IL-$1^{-/-}$ or wild-type mice (Fig. 4), while food intake/body weight after vehicle treatment was not significantly different (data not shown). The responses of IL-$6^{-/-}$ and IL-$6^{-/-}$ IL-$1^{-/-}$ mice were not significantly different each other (Fig. 4). These results indicate that endogenous expression of IL-6 is involved in IL-1–induced anorexia and also suggest the possibility that expression of IL-1 under physiological conditions in IL-$6^{-/-}$ mice reduces food intake and that the absence of endogenous IL-1 expression in IL-$6^{-/-}$ IL-$1^{-/-}$ mice contributes to increase in food intake and development of an obese phenotype.

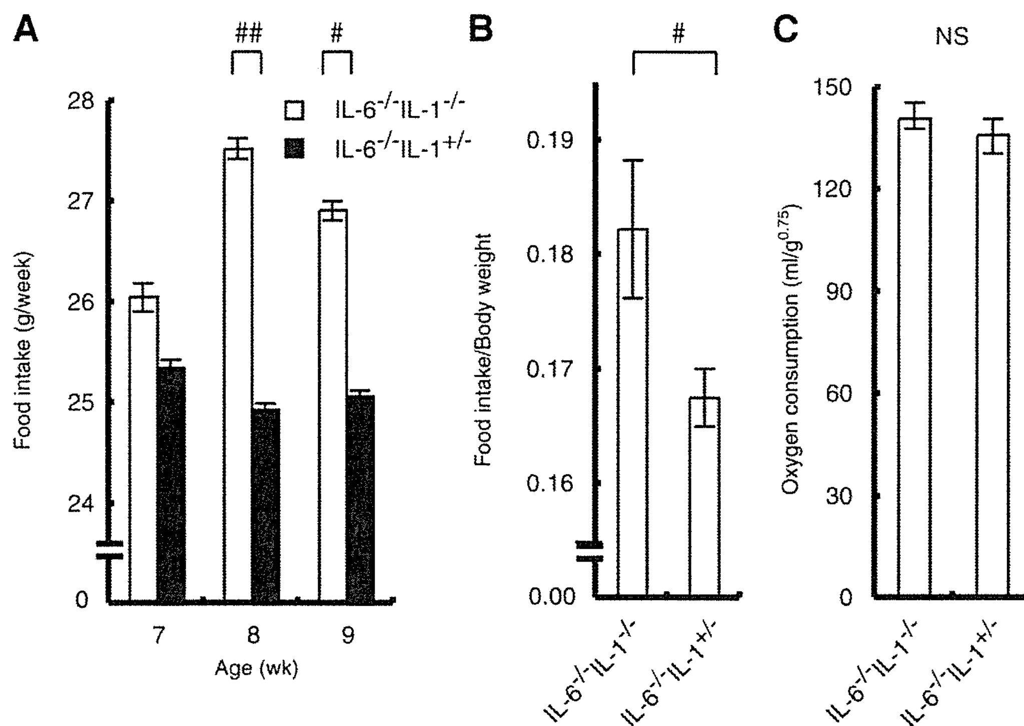


**FIG. 3. Food intake and energy expenditure in IL-6$^{-/-}$ IL-1$^{-/-}$ male mice. *A*: Weekly food intake from 7 to 9 weeks of age in IL-6$^{-/-}$ IL-1$^{-/-}$ ($n$ = 6) and IL-6$^{-/-}$ IL-1$^{+/-}$ mice ($n$ = 8). *B*: Daily food intake per body weight from 7 to 9 weeks in IL-6$^{-/-}$ IL-1$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{+/-}$ mice. Daily food intake per body weight was calculated by dividing the total food intake by the body weight at 7 weeks of age. *C*: Metabolic rate in IL-6$^{-/-}$ IL-1$^{-/-}$ ($n$ = 5) and IL-6$^{-/-}$ IL-1$^{+/-}$ ($n$ = 6) mice. Data are expressed as means ± SE. Statistical significance was calculated by repeated-measures ANOVA and Student's $t$ test (*A*) by Student's $t$ test (*B* and *C*). #$P$ < 0.05; ##$P$ < 0.01; NS, not significant.**

## DISCUSSION

In this study, we examined the effect of IL-1 deficiency on body weight and found that increases in body weight were only observed in mice of the IL-6–deficient background. No differences were observed in single IL-1$^{-/-}$ until 8 months or IL-6$^{-/-}$ mice until 4 months. In contrast, the body weight of IL-6$^{-/-}$ IL-1Ra$^{-/-}$ mice was similar to that of IL-1Ra$^{-/-}$ mice, indicating that excess IL-1 signaling promotes leanness independent of IL-6 signaling. These results indicate that both IL-1 and IL-6 are involved in the regulation of body weight homeostasis, playing compensatory roles through independent pathways. Food intake increased only in IL-6$^{-/-}$ IL-1$^{-/-}$ mice, while IL-1$^{-/-}$ and IL-6$^{-/-}$ mice exhibit normal feeding behavior, suggesting that the activities of these cytokines on body weight in young adult animals are primarily by their effects on feeding behavior. As the acute decreases in food intake induced by IL-1 treatment were significantly suppressed in IL-6$^{-/-}$ IL-1$^{-/-}$ mice in comparison to IL-1$^{-/-}$ and wild-type mice, endogenous IL-1 and IL-6 comprise compensatory mechanisms influencing IL-1–induced decreases in food intake.

Our observations that the leanness in IL-1Ra$^{-/-}$ mice is not dependent on IL-6 and that the obesity in IL-1$^{-/-}$ mice is dependent on IL-6 seem apparently paradoxical. However, these observations could be explained by mutual redundancy of the suppression mechanisms of body fat gain by IL-6 and IL-1 in young mice. Absence of both IL-1 and IL-6 is needed to cancel the suppression, at least in young mice. We have previously demonstrated that food intake in IL-1Ra$^{-/-}$ mice was not significantly different from wild-type mice and that hypoinsulinemia accompanied with increased insulin sensitivity is responsible for leanness in IL-1Ra$^{-/-}$ mice (7). Thus, our observation that the leanness in IL-1Ra$^{-/-}$ mice is not dependent on IL-6 indicates that IL-6 is not responsible for hypoinsulinemia in IL-1Ra$^{-/-}$ mice. In contrast to IL-1Ra$^{-/-}$ mice, we found that food intake in IL-6$^{-/-}$ IL-1$^{-/-}$ mice were increased compared with IL-6$^{-/-}$ IL-1$^{+/-}$ mice. Thus, our observation that the obesity in IL-1$^{-/-}$ mice is dependent on IL-6 indicates that IL-6 is responsible for IL-1–induced suppression of food intake. These effects are only evident in IL-6$^{-/-}$ IL-1$^{-/-}$ mice due to compensatory mechanisms such as local increase of IL-6 or increased sensitivity in IL-6 signaling pathway in IL-1$^{-/-}$ mice. Although we have examined the basal levels of IL-6 in IL-1 knockout mice and of IL-1 in IL-6 knockout mice, we could not find a significant difference in the expression levels in the brain (20), adipose tissue, and skeletal muscle compared with wild-type mice (data not shown), and we could not detect basal levels for serum IL-1 or IL-6 in knockout mice in our enzyme-linked immunosorbent assay system (26) (data not shown). However, it is possible that basal levels of IL-6 and IL-1 could be differently regulated in IL-6$^{-/-}$ and IL-1$^{-/-}$ mice in tissues not examined. The fact that IL-1Ra$^{-/-}$ mice show normal food intake suggests that regulation of IL-1 signaling by IL-1Ra to regulate appetite in hypothalamus have small significance.

The roles of IL-6 on body weight regulation were established by series of studies by Dr. Jansson's group (15,16). They proposed that IL-6 acts on the central nervous system to regulate energy expenditure, because intracerebroventricular injection of IL-6 increased energy expenditure, although basal energy expenditure in IL-6$^{-/-}$ mice did not change compared with wild-type mice. As leptin induces hypothalamic IL-1β expression and intracerebroventricular injection of IL-1Ra suppresses leptin-induced appetite loss (4), it is likely that IL-1 in the brain is responsible for obesity in IL-6$^{-/-}$ IL-1$^{-/-}$ mice (in this study) and leanness in IL-1Ra$^{-/-}$ mice (7). As we found that IL-1–induced suppression of appetite is partially dependent on IL-6 (Fig. 4), IL-1 signaling and IL-6 signaling leading to appetite loss are likely to cross talk within the brain. Although, downstream mediators responsible for increased food intake in IL-6$^{-/-}$ IL-1$^{-/-}$ mice are not clear at present. One possible explanation is leptin resistance. We found that serum leptin levels were significantly increased in IL-6$^{-/-}$ IL-1$^{-/-}$ mice (Fig. 2*F*), indicating poten-

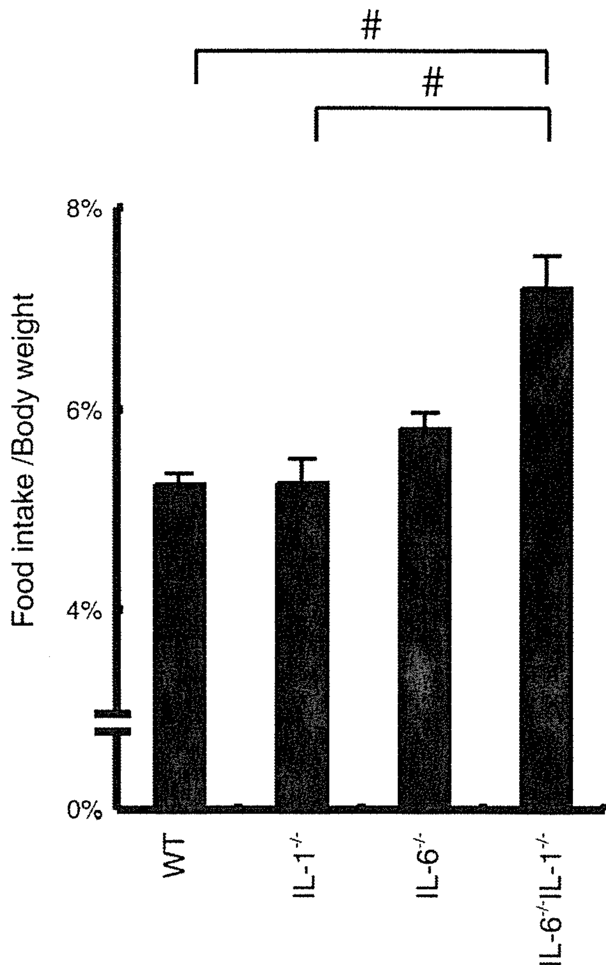


**FIG. 4. Food intake after IL-1α treatment is higher in IL-6$^{-/-}$ IL-1$^{-/-}$ mice. Food intake in wild-type (WT), IL-1$^{-/-}$, IL-6$^{-/-}$, IL-6$^{-/-}$ IL-1$^{-/-}$, and IL-6$^{-/-}$ IL-1$^{+/-}$ mice after IL-1α injection (1 μg/kg body wt i.p.) was calculated by subtracting the 24-h value from the value measured before treatment for each animal. The change ratio was calculated by dividing the resulting value by the body weight of the animal. The following numbers of mice were used: WT ($n$ = 7), IL-1$^{-/-}$ ($n$ = 7), IL-6$^{-/-}$ ($n$ = 9), and IL-6$^{-/-}$IL-1$^{-/-}$ ($n$ = 6) mice. Data are expressed as the means ± SE. Statistical significance was calculated by ANOVA followed by Scheffe's test. #$P$ < 0.05.**

tial leptin resistance in IL-6$^{-/-}$ IL-1$^{-/-}$ mice. It remains unclear, however, whether leptin resistance is the cause or merely the result of obesity in IL-6$^{-/-}$ IL-1$^{-/-}$ mice as leptin levels correlate with fat mass (27). Another possibility is melanocortin peptides, as it was previously demonstrated that MC3/4R antagonist, SHU9119, reversed IL-1β–induced reductions in food intake (28). We analyzed the expression levels for proopiomelanocortin and agouti-related protein in IL-1Ra$^{-/-}$ mice (7) and IL-6$^{-/-}$ IL-1$^{-/-}$ mice, but we could not find a significant difference from wild-type mice (data not shown). Another possibility is neuropeptide Y (NPY), as a chronic IL-1β treatment, reduced expression levels for NPY (29) and NPY blocks and reverses IL-1β–induced anorexia (30). We also analyzed the expression levels for NPY in IL-1Ra$^{-/-}$ mice (7) and IL-6$^{-/-}$ IL-1$^{-/-}$ mice, but we could not find a significant difference from wild-type mice (data not shown). Further studies are required to clarify the downstream mechanisms responsible for increased food intake in IL-6$^{-/-}$ IL-1$^{-/-}$ mice.

The effects of IL-6 on glucose and lipid homeostasis were extensively studied. Several articles demonstrated that IL-6 induces insulin resistance in hepatocytes (31), skeletal muscle (32), and adipose tissue (33) and that IL-6 induces lipolysis and fat oxidation (34). The effect of IL-1 on glucose and lipid homeostasis was also extensively studied. IL-1 induces insulin resistance in hepatocytes (35) and mediates glucotoxicity in pancreatic islets (36). We have previously demonstrated that IL-1Ra knockout mice are hypoinsulinemic accompanied with increased insulin sensitivity (7). We found that insulin levels in IL-6$^{-/-}$ IL-1$^{-/-}$ mice were relatively high but not statistically significant compared with control mice. So higher levels of serum insulin in IL-6$^{-/-}$ IL-1$^{-/-}$ mice may be secondary to obesity. To analyze the possible effect of IL-6 deficiency on glucose and insulin homeostasis directly, we performed glucose tolerance test and insulin tolerance test analysis in IL-6$^{-/-}$ IL-1Ra$^{-/-}$ and IL-6$^{-/-}$ IL-1$^{-/-}$ mice but failed to find any significant difference compared with single deficient mice and wild-type mice in young mice (~16 weeks) (data not shown). These results suggest that the effect of IL-6 is primarily dependent on food intake and that IL-6 is not important for insulin release or insulin sensitivity in IL-1Ra$^{-/-}$ mice.

In a classical view, the role of inflammatory cytokines was restricted under pathological conditions. It is well known that IL-1 and IL-6 are induced in the brain in central nervous system infections or injury (37). It should be noted that the roles of inflammatory cytokines, including IL-6 and IL-1 under inflammatory conditions, might be different from those under physiological conditions. In this study, we have demonstrated that IL-1 and IL-6 expressed under physiological conditions reduce food intake to regulate body weight. Recently, it was suggested that obesity is associated with a state of chronic, low-grade inflammation in adipocytes (38,39) and hepatocytes (40,41). Inflammatory cytokines, including IL-1, tumor necrosis factor-α, and IL-6, are induced in adipocytes and hepatocyte, which occurs concomitant with the infiltration of macrophages into adipose and hepatocyte of severe obese mice. Further studies are required for the role of IL-1 signaling under severe obesity.

As human life has always suffered from not only physical stress such as infections or injuries but also mental stresses, which induce inflammatory cytokines in the brain, the role of inflammatory cytokines in obesity and diabetes is potentially of great importance to human obesity. Further analysis of the role of inflammatory cytokines in obesity and diabetes will shed light on new therapeutic strategies for the treatment of metabolic diseases.

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# IL-1β, but not IL-1α, is required for antigen-specific T cell activation and the induction of local inflammation in the delayed-type hypersensitivity responses

**Aya Nambu, Susumu Nakae**[1] and **Yoichiro Iwakura**

Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

[1]Present address: Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5176, USA

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**Abstract**

**As IL-1 expression is augmented in delayed-type hypersensitivity (DTH) responses, we analyzed the role of IL-1 in this response. DTH responses against methyl BSA (mBSA) were significantly suppressed in IL-1β-deficient (IL-1β$^{-/-}$) and IL-1α/β$^{-/-}$ mice, but not in IL-1α$^{-/-}$ mice. In contrast, responses in IL-1R antagonist$^{-/-}$ (IL-1Ra$^{-/-}$) mice were exacerbated. Lymph node cells derived from mBSA-sensitized IL-1β$^{-/-}$, IL-1α/β$^{-/-}$ and IL-1R type I (IL-1RI)$^{-/-}$ mice, but not from IL-1α$^{-/-}$ mice, exhibited reduced proliferative responses against mBSA, while these from IL-1Ra$^{-/-}$ mice demonstrated augmented responses. DTH responses in wild-type mice following adoptive transfer of CD4$^+$ T cells from mBSA-sensitized IL-1α/β$^{-/-}$ mice were also reduced, while those in mice given cells derived from IL-Ra$^{-/-}$ mice were increased. DTH responses in IL-1RI$^{-/-}$, but not IL-1α/β$^{-/-}$, mice were reduced upon transplantation of mBSA-sensitized CD4$^+$ T cells from wild-type mice. The recall response of mBSA-sensitized CD4$^+$ T cells against mBSA decreased upon co-culture with dendritic cells (DCs) from IL-1RI$^{-/-}$ mice, while the responses were normal with DCs from IL-1α/β$^{-/-}$ mice. DTH responses in tumor necrosis factor α$^{-/-}$ (TNF$^{-/-}$) mice were also suppressed; the magnitude of the suppression in IL-1α/β$^{-/-}$TNF$^{-/-}$ mice, however, was similar to that observed in IL-1α/β$^{-/-}$ mice. These observations indicate that IL-1 possesses dual functions during the DTH response. IL-1β is necessary for the efficient priming of T cells. In addition, CD4$^+$ T cell-derived IL-1 plays an important role in the activation of DCs during the elicitation phase, resulting in the production of TNF, that activate allergen-specific T cells.**

## Introduction

Delayed-type hypersensitivity (DTH), classified as type IV hypersensitiveness by Coombs and Gell, is an immune response mediated by a variety of inflammatory cells, including neutrophils, macrophages and T cells (1, 2). DTH develops in two phases, a sensitization phase, in which T cells are sensitized and memory T cells are formed, and an elicitation phase, in which T cell recall responses are induced upon secondary challenge with antigens. This second phase results in the induction of inflammation, involving recruitment of inflammatory cells such as neutrophils and macrophages.

Although DTH reactions are classically subdivided into tuberculin-type, Jones–Mote-type and contact hypersensitivity (CHS) reactions, in this study, we analyzed the mechanisms of tuberculin-type reaction as typical of DTH reactions. The DTH response is evoked by $T_h1$ CD4$^+$ T cells, whereas CD8$^+$ T cells behave as apparent regulatory cells in this response (3). During the elicitation phase of DTH responses, neutrophils and macrophages infiltrate early into inflammatory sites, followed by T cells. IFN-γ, produced by antigen-specific CD4$^+$ T cells, plays an important role in the development of DTH responses by enhancing $T_h1$ cell development (4), leukocyte recruitment through the induction of chemokines, such as IFN-γ-inducible protein-10 (IP-10) (5), and expression of adhesion molecules, such as intercellular adhesion

*Correspondence to*: Y. Iwakura; E-mail: iwakura@ims.u-tokyo.ac.jp
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molecule-1, vascular cell adhesion molecule-1, E-selectin and P-selectin by acting on leukocytes, endothelial cells and keratinocytes (6–9). IFN-γ also activates macrophages, resulting in the production of IL-1 and tumor necrosis factor α (TNFα), factors that perpetuate the local inflammation (10).

IL-1 is a pro-inflammatory cytokine that regulates multiple aspects of immune and inflammatory responses (11). The IL-1 family of cytokines includes IL-1α and IL-1β. While these molecules both bind to two cellular receptors, IL-1R type I and type II (IL-1RI and IL-1RII, respectively) (12), the roles for these cytokines differ between types of host defense responses (13). IL-1RI, but not IL-1RII, mediates the functional activities of IL-1α and IL-1β (12). Thus, IL-1RII is considered to be a decoy receptor that negatively regulates IL-1 activity (12). IL-1R antagonist (IL-1Ra), also a negative regulator of IL-1 function, can bind to IL-1RI without generating any signal, competing for IL-1 binding and negatively regulating IL-1α and IL-1β signaling (12).

IL-1 contributes to the development of DTH responses by promoting the maturation of antigen-specific T cells (14) and by augmenting IL-12-dependent IFN-γ production by $T_h1$ cells (15). The development of DTH responses were reduced in mice lacking the expression of IL-1RI (IL-1RI$^{-/-}$ mice) (16) or treated with peptides derived from IL-1Ra (17). It has also been reported, however, that IL-1β$^{-/-}$ mice exhibit normal DTH responses (18), suggesting that IL-1α, but not IL-1β, contributes to the generation of type IV hypersensitivity reactions.

Recently, we have demonstrated that IL-1α, not IL-1β, potently activates T cells during the sensitization phase of CHS responses (19). The role of IL-1 in DTH responses, however, remains unclear; it is unknown whether IL-1 acts as a T cell-activating factor during the sensitization phase or as a pro-inflammatory cytokine recruiting inflammatory cells during the elicitation phase.

In this study, we have analyzed the activities of IL-1 and the functional differences between IL-1α and IL-1β in the development of DTH responses against methyl BSA (mBSA). We demonstrate that IL-1β, but not IL-1α, functions in the development of DTH responses using IL-1α-, IL-1β-, IL-1α/β-, IL-1RI- and IL-1Ra-deficient mice. We also demonstrate that, in addition to the involvement of IL-1 in T cell sensitization, activated memory T cell-derived IL-1 also induces dendritic cell (DC)-mediated production of TNF, resulting in local inflammation upon secondary stimulation.

## Methods

### *Mice*

IL-1α$^{-/-}$, IL-1β$^{-/-}$, IL-1α/β$^{-/-}$ and IL-1Ra$^{-/-}$ mice were generated as described (20). Animals were backcrossed to either C57BL/6J or BALB/cA mice for eight generations. IL-1RI$^{-/-}$ mice, kindly provided by Immunex (21), were backcrossed to C57BL/6J mice for six generations. TNF$^{-/-}$ mice were backcrossed for 10 generations to C57BL/6J mice (22). IL-1α/β$^{-/-}$TNF$^{-/-}$ mice and IL-1Ra$^{-/-}$TNF$^{-/-}$ mice were obtained by intercrossing IL-1α/β$^{-/-}$ and TNF$^{-/-}$ mice and IL-1Ra$^{-/-}$ and TNF$^{-/-}$ mice, respectively. C57BL/6J and BALB/cA mice were purchased from CLEA-Japan (Shizuoka, Japan). Sex- and age-matched mice, used in all experiments, received simultaneous treatment. All mice were housed under specific pathogen-free conditions in an environmentally controlled clean room (24 ± 2°C, 40–60% moisture, 8:00–20:00 lighting cycle) at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. Animals were periodically monitored for infection. Experiments were conducted according to the institutional ethical guidelines for animal experimentation and the safety guidelines for gene manipulation experiments.

### *DTH response*

Evaluation of DTH responses to mBSA (Sigma, St Louis, MO, USA) was performed as described (23–25). Briefly, mice were immunized subcutaneously (s.c.) with 200 μl of 1.25 mg ml$^{-1}$ mBSA emulsified with CFA (Difco, Detoit, MI, USA). Seven days after immunization, mice were challenged s.c. in one footpad with 20 μl of 10 mg ml$^{-1}$ mBSA in PBS. Animals were injected with an equal volume of PBS into another footpad as a control. At the indicated times after the challenge, footpad thickness was measured with a dial caliper (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as follows: [footpad swelling (mm)] = [footpad thickness of mBSA-injected footpad (mm)] − [footpad thickness of PBS-injected footpad (mm)], [footpad swelling (%)] = ([footpad swelling (mm)]/[footpad thickness of PBS-injected footpad (mm)]) × 100. Footpad swelling (%) was calculated as a percentage of the mean value of the wild-type group.

### *Histological examination*

Twenty-four hours after challenge with either mBSA or PBS, paws were removed and decalcified for 3 days by a standard protocol. After fixation with 10% PFA in PBS, specimens were embedded in paraffin. Paraffin sections were stained with hematoxylin–eosin. Structural changes and the presence of cellular infiltration in sections were determined by light microscopy.

### *Proliferative responses of lymph node cells*

Mice were immunized with 250 μg (when IL-1$^{-/-}$ or IL-1RI$^{-/-}$ mice were immunized) or 100 μg (when IL-1Ra$^{-/-}$ mice were immunized) mBSA/CFA at the base of the tail and two footpads. Seven days after immunization, inguinal and popliteal lymph nodes (LN) were harvested. After preparation of a single cell suspension, LN cells were suspended in RPMI1640 (Sigma) supplemented with 10% FCS (Sigma), 50 U ml$^{-1}$ penicillin (Meiji), 50 μg ml$^{-1}$ streptomycin (Meiji) and 50 mM 2-mercaptoethanol (GIBCO). LN cells (5 × $10^5$) were cultured in the presence or absence of mBSA (50 μg ml$^{-1}$) in 96-well plates for 72 h, followed by the incorporation of [$^3$H]thymidine ([$^3$H]TdR) (0.25 mCi ml$^{-1}$) (Amersham, Buckinghamshire, UK) for 9 h. Cells were then harvested using a Micro 96 well harvester (Molecular Devices, Sunnyvale, CA, USA); radioactivity was measured using a Micro Beta counter (Amersham).

### *Measurement of IFN-γ production*

IFN-γ levels in LN cell culture supernatants were determined by sandwich ELISA. IFN-γ Opt EIA kit (BD Biosciences Pharmingen, San Diego, CA, USA) was used for the detection

of IFN-γ. Resulting absolute cytokine levels were determined according to the manufacturer's protocol. The detection limit of the assay for IFN-γ was >5 pg $ml^{-1}$.

*Measurement of serum anti-mBSA IgG*

Sera were collected prior to immunization with mBSA/CFA and 4 days after the second immunization with DTH. Anti-mBSA IgG levels were measured by ELISA (25). Briefly, 96-well plates were coated overnight with 100 μl of a 5 μg $ml^{-1}$ mBSA solution at 4°C, then blocked by incubation with 0.1% skim milk (CO-OP, Tokyo, Japan) in PBS for 1 h at room temperature (r.t.). After washing three times in PBS + 0.05% Tween 20, 100 μl of each mouse serum, diluted 316-fold in PBS, was added for 1 h at r.t.. After another round of extensive washing, we added 100 μl of alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (ZYMED, San Francisco, CA, USA). After a 1-h incubation at r.t., plates were washed three times. AP activities were measured on a microplate-reader MP-120 (CORONA, Ibaragi, Japan) using *p*-nitrophenylphosphate SIGMA104® (Sigma) as the substrate.

*Adoptive transfer of sensitized T cells*

For adoptive T cell transfer experiments, LN cell suspensions were prepared from mBSA/CFA-sensitized mice as described above. To purify $CD4^+$ T cells, LN cells were incubated with anti-mouse B220, CD8, Ter-119 and NK-1.1 microbeads (Miltenyi Biotech, Auburn, CA, USA). $CD4^+$ T cells were isolated following negative selection on a MACS column. We injected the purified $CD4^+$ T cells ($2 \times 10^7$ cells per mouse) into the tail vain of recipient mice. After 19 h, mice were challenged with mBSA or PBS only into the left or right footpad, respectively. Swelling was monitored over the following days.

*mBSA-specific $CD4^+$ T cell proliferation*

$CD4^+$ T cells were purified (>90% $CD4^+$ cells) from the draining LNs of immunized mice by positive selection using anti-CD4 magnetic beads (MACS; Miltenyi Biotech). $CD11c^+$ cells (DCs) were purified (>90% $CD11c^+$ cells) from non-immunized splenocytes using anti-CD11c magnetic beads (MACS; Miltenyi Biotech). $CD4^+$ T cells ($1 \times 10^5$) were cultured for 72 h with $1 \times 10^4$ DCs in the presence or absence of 50 μg $ml^{-1}$ mBSA in RPMI1640 (Sigma) complete medium. Proliferation was assessed by measurement of [$^3$H]TdR (0.25 mCi $ml^{-1}$) (Amersham) incorporation.

*Reverse transcription–PCR*

Total RNA from $CD4^+$ T cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method. For reverse transcription (RT), 5 μg of total RNA was transcribed using SuperScript III transcriptase according to the manufacturer's protocol of SuperScript first-strand synthesis system for RT–PCR (Invitrogen, San Diego, CA, USA). The PCR mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, a 5′ and 3′ primer (each at 0.2 μM), 2 units Ex *Taq* DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and 5 μl (10% of total volume) of the products of RNA reverse transcription in a total volume of 50 μl. After an initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C for 20 s), annealing (55°C for 30 s) and extension (72°C for 45 s) were performed using a DNA thermal cycler (icycler, Bio-Rad, Hercules, CA, USA). The primers for PCR amplifying IL-1α, IL-1β, IL-1RI, IL-1Ra and hypoxanthine phosphoribosyltransferase (HPRT) are as follows: forward, 5′-GAGATACAAACTGATGAAGCTC-3′, and reverse, 5′-CAGAAGAAAATGAGGTCGGTC-3′ (IL-1α); forward, 5′-CCTGAACTCAACTGTGAAATGCC-3′, and reverse, 5′-TCATCATCATCCCATGAGTCAC-3′ (IL-1β); forward, 5′-CTGTAAACCTCTGCTTCTTGAC-3′, and reverse, 5′-ACAACACAGATAAACGGATAGCG-3′ (IL-1RI); forward, 5′-GACCCTGCAAGATGCAAGCC-3′, and reverse, 5′-GAGCGGATGAAGGTAAAGCG-3′ (IL-1Ra); forward, 5′-GTTGGATACAGGCCAGACT-3′, and reverse, 5′-CAGGGTAGGCTGGCCTATAGGCT-3′ (HPRT).

*Statistical analysis*

Each experiment was repeated at least three times. Statistical analysis was performed using the Student's *t*-test. A mean ± SD is shown for all figures. *P*-values <0.05 were considered to be statistically significant.

## Results

*DTH response in IL-1 KO mice*

To elucidate the role of IL-1 in DTH responses, we examined mBSA-induced hypersensitivity in IL-$1^{-/-}$ mice. Footpad swelling in IL-1α/$β^{-/-}$ mice on the C57BL/6J background was significantly reduced compared with that seen in wild-type mice (Fig. 1A). A similar reduction was also observed in IL-$1RI^{-/-}$ mice, consistent with the results of Labow *et al.* (16). In contrast, footpad swelling in IL-$1Ra^{-/-}$ mice was markedly enhanced (Fig. 1B). Similar observations were obtained in IL-1α/$β^{-/-}$ mice on the BALB/cA background (data not shown).

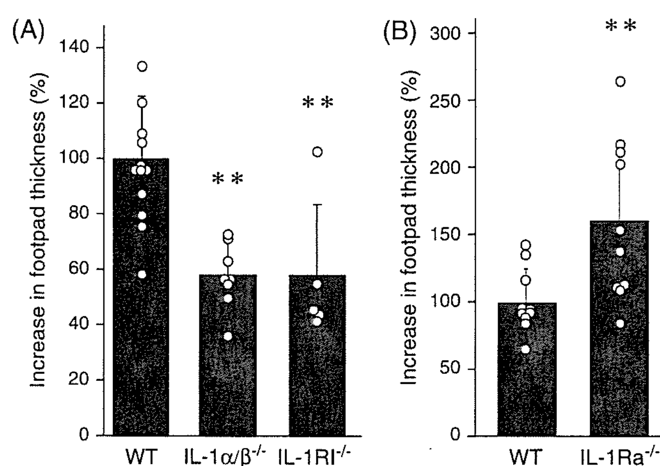


**Fig. 1.** Induction of DTH responses in IL-1α/$β^{-/-}$, IL-$1RI^{-/-}$ and IL-$1Ra^{-/-}$ mice. Mice were sensitized s.c. with mBSA/CFA (250 μg in A, 100 μg in B). One week after sensitization, PBS or mBSA was injected into the footpads of each mouse. Footpad thickness was measured 24 h after the second challenge. Circles indicate the levels for individual mice; a bar shows the mean ± SD for each group. The numbers of the mice used were (A) wild type, $n = 12$; IL-1α/$β^{-/-}$, $n = 7$ and IL-$1RI^{-/-}$, $n = 5$; (B) wild type, $n = 8$, and IL-$1Ra^{-/-}$, $n = 4$. **$P < 0.01$ versus wild-type mice.

In the mBSA-injected footpads of wild-type mice, we observed the infiltration of inflammatory cells mainly consisting of neutrophils and lymphocytes in the dermis surrounding small vessels; these infiltrates were not observed in PBS-injected footpads (Fig. 2A–D). Consistent with the reduced footpad thickness (Fig. 1), the numbers of infiltrating cells in the mBSA-challenged footpads of IL-1RI$^{-/-}$ and IL-1α/β$^{-/-}$ mice (Fig. 2F and G) were significantly lower than those in wild-type mice, although these levels were increased in comparison with those seen in PBS-injected footpads (Fig. 2B and C). In contrast, a greater number of inflammatory cells were observed in the mBSA-injected footpads of IL-1Ra$^{-/-}$ mice than that seen in wild-type mice (Fig. 2H). The cellular composition infiltrated into the inflammatory sites was similar (data not shown) among IL-1α/β$^{-/-}$, IL-1RI$^{-/-}$ and IL-1Ra$^{-/-}$ mice (Fig. 2I–L). These results indicate that IL-1 plays an important role in the development of mBSA-induced DTH responses.

*The role for IL-1 in the activation of mBSA-specific T cells*

As IL-1 functions as a T cell co-stimulatory molecule in the antigen-specific T cell activation observed during the sensitization phase of immune responses (19, 26), we examined the effect of IL-1 deficiency on antigen-specific T cell responses following mBSA sensitization. Seven days after mBSA sensitization, isolated inguinal LN cells were cultured in the presence or absence of mBSA. LN T cell proliferative responses against mBSA were reduced in both IL-1RI$^{-/-}$ and IL-1α/β$^{-/-}$ mice from the levels seen in wild-type mice (Fig. 3A). Under these conditions, IFN-γ levels in the supernatants of LN cell cultures derived from IL-1RI$^{-/-}$ and IL-1α/β$^{-/-}$ mice were decreased in comparison with those observed in LN cell cultures from wild-type mice (Fig. 3B). The levels of IL-4 in these LN cell cultures, however, were below the assay limit of the detection among these LN cell cultures (data not shown). The mBSA-specific proliferative responses of LN cells from IL-1Ra$^{-/-}$ mice were increased 2.2-fold from those seen in cultures from wild-type mice (Fig. 3C); the IFN-γ levels in supernatants from IL-1Ra$^{-/-}$ LN cell cultures were also elevated (Fig. 3D). These results indicate that IL-1 is required for optimal T cell activation during the sensitization phase of DTH responses induced by mBSA.

*Differential roles of IL-1α and IL-1β in DTH response*

To clarify the differential roles of IL-1α and IL-1β in the development of DTH responses, we have examined the expression of IL-1α and IL-1β during DTH reaction. Mice were immunized

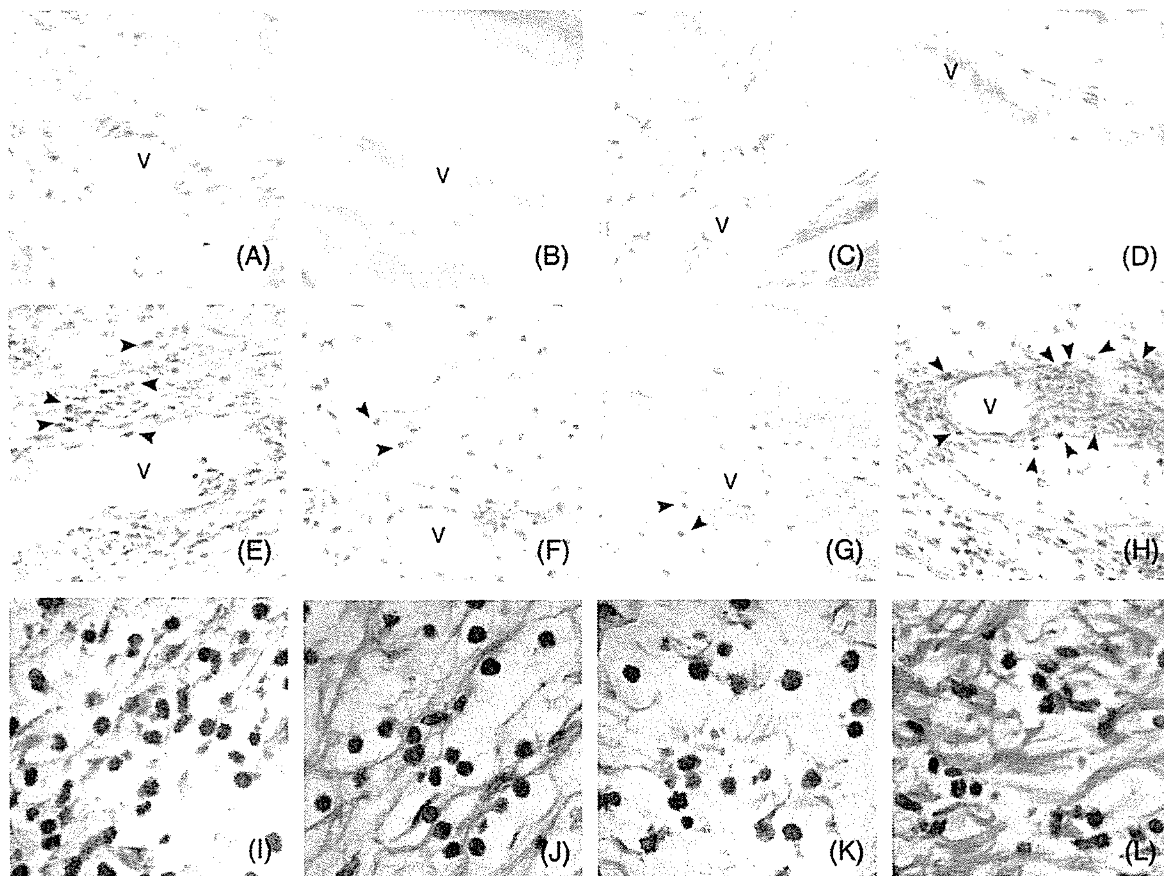


**Fig. 2.** Histology of footpads from DTH-induced mice. Sections of footpads challenged with PBS (A, B, C and D) or mBSA (E–H) were stained with hematoxylin and eosin. (A) and (E), wild-type mice; (B) and (F), IL-1α/β$^{-/-}$ mice; (C) and (G), IL-1RI$^{-/-}$ mice (D) and (H), IL-1Ra$^{-/-}$ mice. "v" points to small vessels. Filled arrow heads in (E–H) indicate infiltration of inflammatory cells in the dermis surrounding small vessels. (A–H): ×400 and (I–L): ×1000

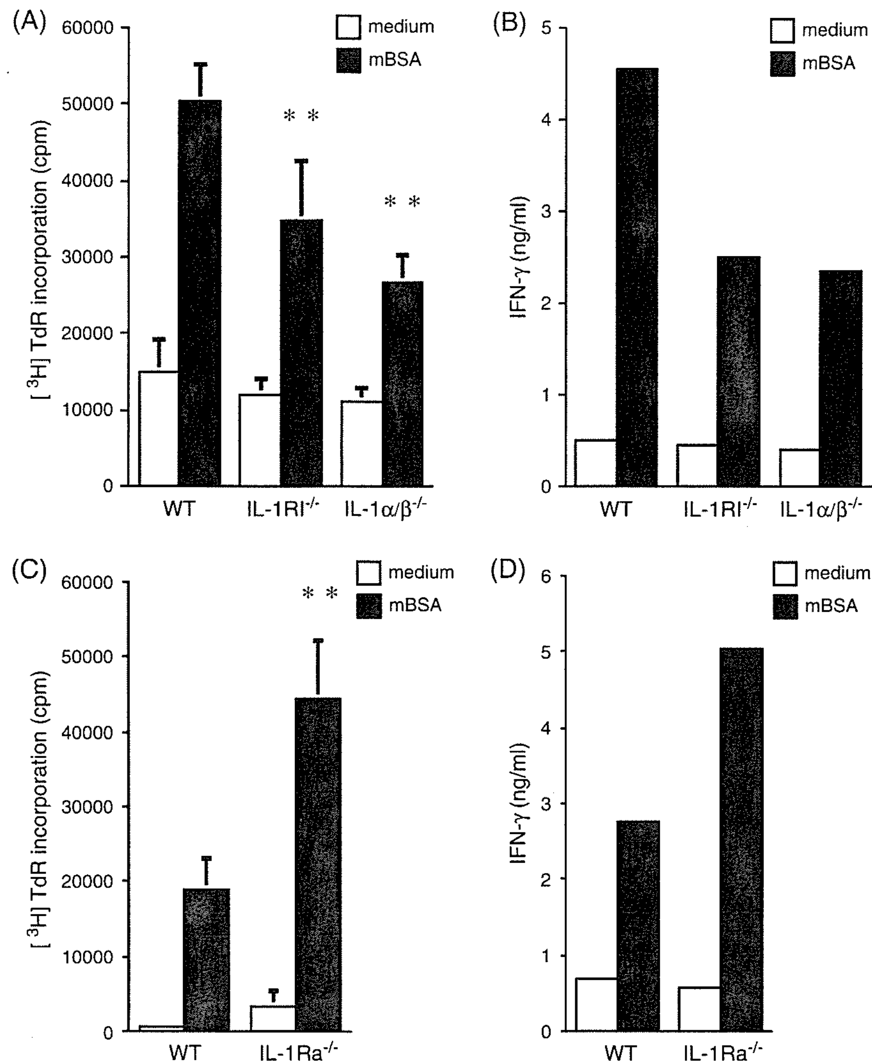


**Fig. 3.** Proliferative responses and IFN-γ production of LN cells after stimulation with mBSA. Mice were immunized with mBSA/CFA (250 μg in A, B, D; 100 μg in C) as described in Methods. (A), (C) Seven days after immunization, LN cells were harvested. After stimulation with mBSA for 72 h, proliferative responses were measured by [$^3$H]TdR incorporation. A mean ± SD of triplicate measurements is shown. (B), (D) IFN-γ levels in the pooled culture supernatants of triplicate wells from proliferative response assay were determined by ELISA. Similar results were obtained at least in three independent experiments. $^{**}P < 0.01$ versus wild-type mice.

with mBSA/CFA, and after 3 days (for the sensitization phase) or 7 days (for the elicitation phase), the LN cells were harvested. Then, the mRNA levels for IL-1α and IL-1β were determined by real-time PCR. We found that the IL-1β expression level in the LN cells was much higher than that of IL-1α both at the sensitization and elicitation phases (the content of IL-1β mRNA in total IL-1 mRNA: 90% at 3 days after sensitization and 65% at 7 days). The expression level of IL-1β in the inflamed footpads at the elicitation phase was also much higher than that of IL-1α (80% IL-1β). Thus, IL-1β is mainly produced during DTH responses.

Then, we examined mBSA-induced hypersensitivity in IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice on the C57BL/6J background. Footpad swelling differed markedly among IL-1α$^{-/-}$, IL-1β$^{-/-}$ and wild-type mice; swelling was significantly suppressed in IL-1β$^{-/-}$ and IL-1α/β$^{-/-}$ mice, while it was unaffected in IL-1α$^{-/-}$ mice (Fig. 4A). Microscopic analyses coincided well with these observations (data not shown). Similar results were also observed in mice on the BALB/cA background (data not shown).

The mBSA-specific proliferative responses of LN cells after sensitization with mBSA were significantly reduced in both IL-1β$^{-/-}$ and IL-1α/β$^{-/-}$ mice in comparison with IL-1α$^{-/-}$ and wild-type mice (Fig. 4B). No significant differences were observed between wild-type mice and IL-1α$^{-/-}$ mice. Interestingly, upon stimulation with mBSA, IFN-γ levels in the supernatants of LN cell cultures prepared from both IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice were decreased from the levels observed in wild-type mice (Fig. 4C). After antigen stimulation, IFN-γ production in IL-1α/β$^{-/-}$ LN cell cultures was significantly reduced in comparison with IL-1β$^{-/-}$ cell cultures. Although IL-1α did not enhance the proliferative responses of LN cells, these findings suggest that both IL-1α and IL-1β are involved in the production of IFN-γ.

We next examined mBSA-specific antibody production in IL-1-deficient mice. At 4 days after the second challenge

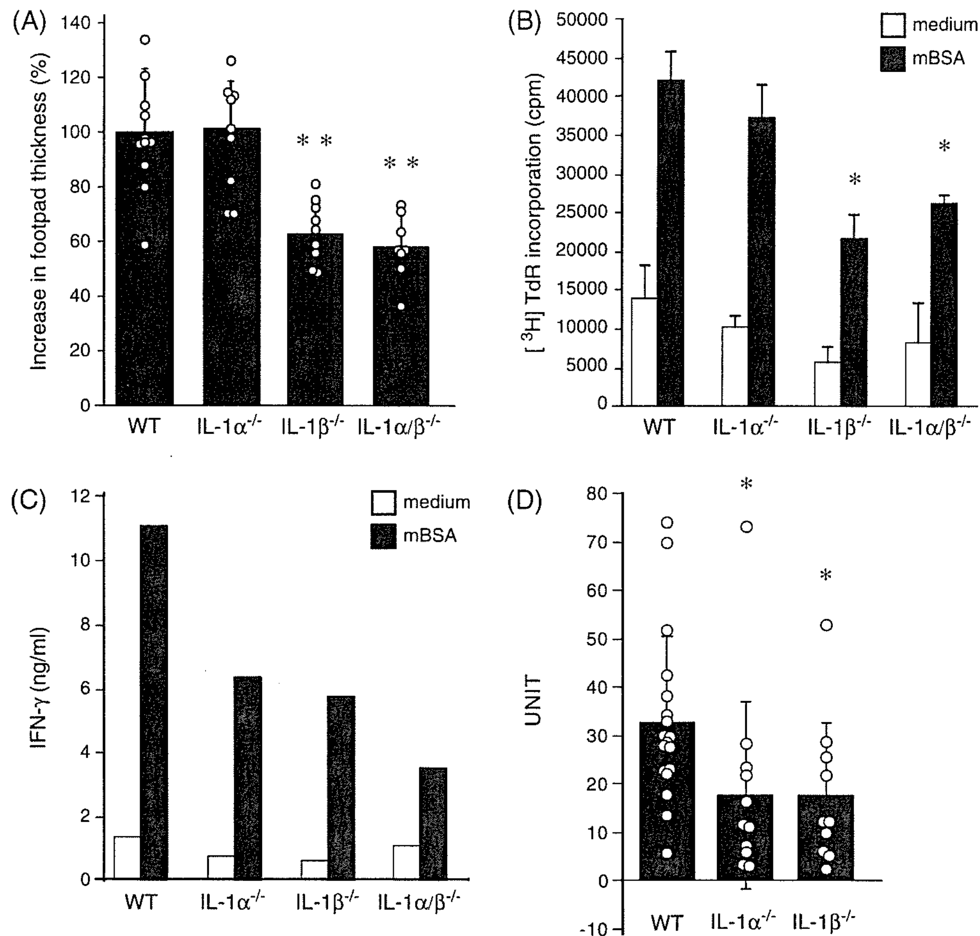


**Fig. 4.** DTH responses in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice. mBSA-induced DTH responses in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice were examined as in Fig. 1. (A) Circles indicate the severity of DTH of individual mice. The mean ± SD of each group is shown. Wild type, $n = 11$; IL-1$\alpha^{-/-}$, $n = 9$; IL-1$\beta^{-/-}$, $n = 9$ and IL-1$\alpha\beta^{-/-}$, $n = 8$. $^{**}P < 0.01$ versus wild-type mice. (B) mBSA-specific LN cell proliferation was assessed by [$^3$H]TdR incorporation. A mean ± SD of triplicate measurements is shown. $^{*}P < 0.05$ versus wild-type mice. (C) IFN-γ levels in pooled culture supernatants of the triplicate wells from the proliferative response assay were determined by ELISA. These results were reproducible in three independent experiments. (D) mBSA-specific IgG levels in sera from DTH-induced mice were determined by ELISA. Circles indicate the levels observed in individual mice. The mean ± SD of each group is also shown. Wild type, $n = 18$; IL-1$\alpha^{-/-}$, $n = 11$ and IL-1$\beta^{-/-}$, $n = 10$. $^{*}P < 0.05$ versus wild-type mice.

with mBSA, mBSA-specific IgG levels in sera were determined by ELISA during the DTH response. mBSA-specific IgG levels were reduced both in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice in comparison with those observed in wild-type mice (Fig. 4D). The DTH response was only suppressed in IL-1$\beta^{-/-}$ mice. Thus, both IL-1α and IL-1β are involved in mBSA-specific antibody production. These results suggested, however, that these antibodies may not be involved in DTH responses.

### *An important role for IL-1 in mBSA-specific T cell sensitization in vivo*

To discriminate the roles of IL-1 in the sensitization of T cells from its function in the elicitation of inflammation, we performed the adoptive transfer of mBSA-sensitized T cells. Mice were first immunized with mBSA/CFA. One week after immunization, we purified T cells from the draining LNs of these mice. These cells were injected intravenously into naive wild-type mice. Measurement of the development of DTH revealed that footpad swelling was significantly suppressed in mice that received mBSA-sensitized IL-1$\alpha/\beta^{-/-}$ T cells in comparison with animals that were given wild-type T cells (Fig. 5A). In contrast, footpad swelling in mice that received mBSA-sensitized IL-1Ra$^{-/-}$ T cells was significantly increased (Fig. 5B). These results indicate that IL-1 is required for antigen-specific T cell priming during the sensitization phase of mBSA-induced DTH responses.

### *The role for IL-1 in the elicitation of DTH response*

To elucidate the role of IL-1 in the elicitation phase of DTH responses, we adoptively transferred mBSA-sensitized wild-type T cells into wild-type, IL-1$\alpha/\beta^{-/-}$, IL-1RI$^{-/-}$ and IL-1Ra$^{-/-}$ mice. We observed a similar footpad swelling in wild-type and IL-1$\alpha/\beta^{-/-}$ mice, while the resulting footpad swelling in IL-1RI$^{-/-}$ mice was markedly decreased (Fig. 5C). Footpad swelling in IL-1Ra$^{-/-}$ mice receiving mBSA-sensitized T cells was significantly increased in comparison to that seen in wild-type recipient mice (Fig. 5D). These results suggest that donor T cell-produced IL-1 activates recipient leukocytes to induce inflammation. In support for this notion, both IL-1α and IL-1β were transiently expressed in sensitized CD4$^+$ T cells

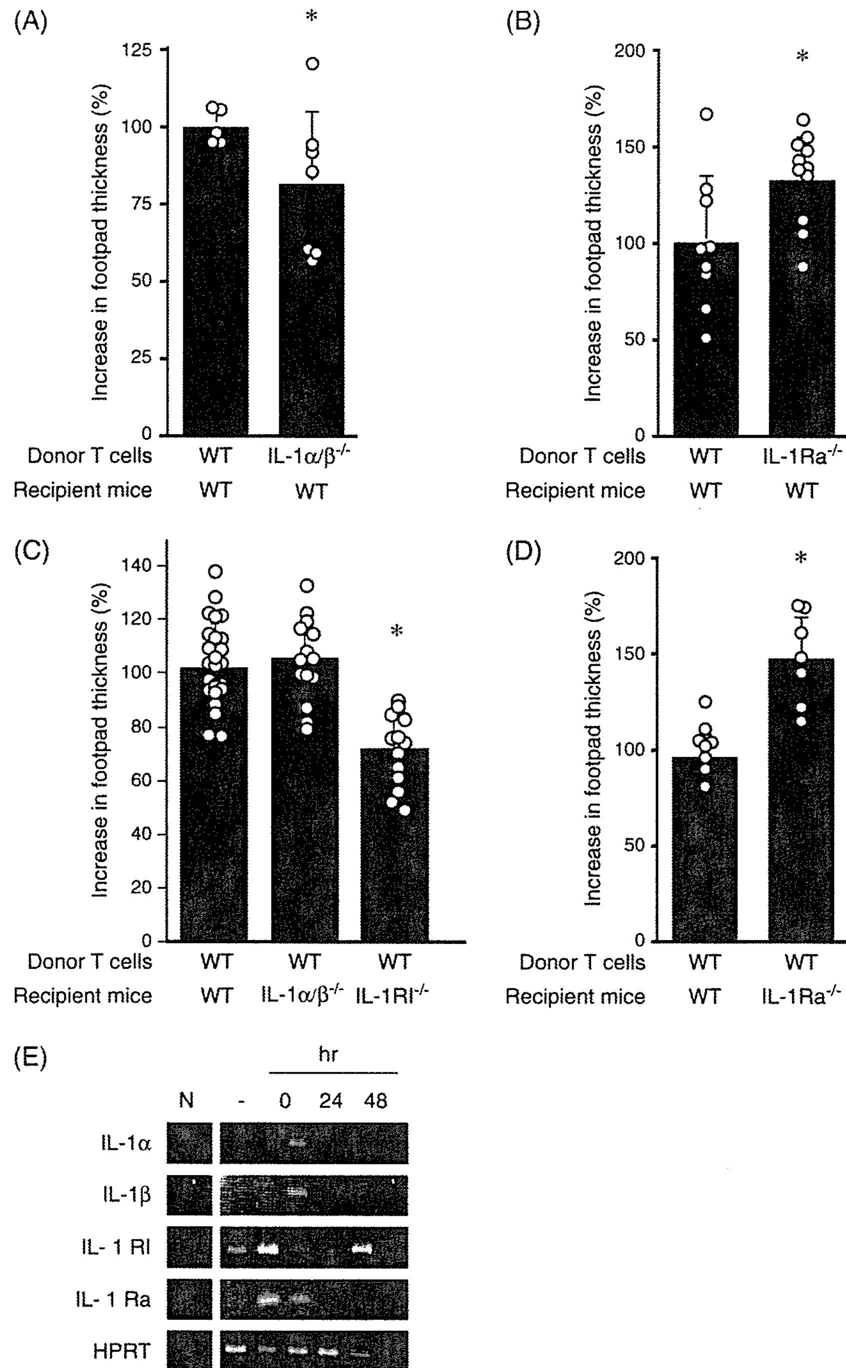


**Fig. 5.** Analysis of the role of IL-1 in the sensitization and elicitation phases of DTH responses. LN cells ($2 \times 10^7$ cells per mouse) from mice immunized with mBSA/CFA were transferred into non-sensitized mice intravenously. Nineteen hours after injection, recipient mice were challenged with PBS or mBSA in footpads. Footpad swelling was measured as in Fig. 1. (A) Wild-type mice received wild-type T cells ($n = 5$) or IL-1$\alpha/\beta^{-/-}$ T cells ($n = 6$), (B) wild-type mice received wild-type T cells ($n = 9$) or IL-1Ra$^{-/-}$ T cells ($n = 12$), (C) wild-type mice received wild-type T cells ($n = 23$), IL-1$\alpha/\beta^{-/-}$ mice received wild-type T cells ($n = 14$) or IL-1RI$^{-/-}$ mice received wild-type T cells ($n = 13$), (D) wild-type mice received wild-type T cells ($n = 10$) or IL-1Ra$^{-/-}$ mice received wild-type T cells ($n = 10$). Circles indicate the levels for individual mice. The mean ± SD of each group is also shown. *$P < 0.05$ versus wild-type mice. (E) Mice were immunized with mBSA or left untreated. After 7 days, CD4$^+$ T cells were purified and cultured for 0, 24 or 48 h. IL-1α, IL-1β, IL-1RI and IL-1Ra mRNA expression were examined by RT–PCR. "–," non-immunized mice; N, without RT. HPRT mRNA levels served as an internal control.

after a 24-h co-culture with mBSA (Fig. 5E). These observations indicate that IL-1, primarily that derived from T cells, is responsible for the induction of local inflammation during the elicitation phase of DTH responses.

*An important role for IL-1 in the activation of DCs during elicitation phase*

We next analyzed the effect of T cell-derived IL-1 on the activation of antigen-presenting cells (APCs) during the

elicitation phase. We isolated CD4$^+$ T cells 7 days after the immunization of wild-type mice with mBSA. Purified cells were co-cultured with DCs from naive wild-type, IL-1α/β$^{-/-}$, IL-1RI$^{-/-}$ and IL-1Ra$^{-/-}$ mice in the presence or absence of mBSA. mBSA-specific CD4$^+$ T cell proliferative responses were significantly reduced following co-culture with IL-1RI$^{-/-}$ DCs, while co-cultures with IL-1α/β$^{-/-}$ DCs generated similar proliferation as that observed in wild-type DCs (Fig. 6A). T cell proliferation in the presence of IL-1Ra$^{-/-}$ DCs, however, was up-regulated (Fig. 6B). These results indicate that T cell-derived IL-1 is important for DC activation, a process that is critical for the subsequent proliferation of antigen-specific CD4$^+$ T cells.

*Involvement of TNF downstream of IL-1 in the DTH response*

TNF is a potent pro-inflammatory cytokine with similar biological activities to IL-1. As IL-1 deficiency only partially reduced the DTH response induced by mBSA (Fig. 1) and IL-1-deficient DCs could activate memory T cells in recall responses (Fig. 6), we examined the possible involvement of TNF in the development of type IV hypersensitivity. Footpad swelling of TNF$^{-/-}$ mice was significantly, if only slightly, reduced (~80%) from the levels seen in wild-type mice (Fig. 7A), suggesting the involvement of TNF in DTH responses. Footpad swelling in IL-1α/β$^{-/-}$TNF$^{-/-}$ mice, however, was similar to that seen in IL-1α/β$^{-/-}$ mice (~60%) (Fig. 7A). No additional effect of TNF deficiency on DTH response could be observed in IL-1α/β$^{-/-}$ mice. The footpad swelling of IL-1Ra$^{-/-}$TNF$^{-/-}$ mice, however, was markedly reduced in comparison with that of IL-1Ra$^{-/-}$ mice (Fig. 7B). These results are compatible with the hypothesis that TNF is

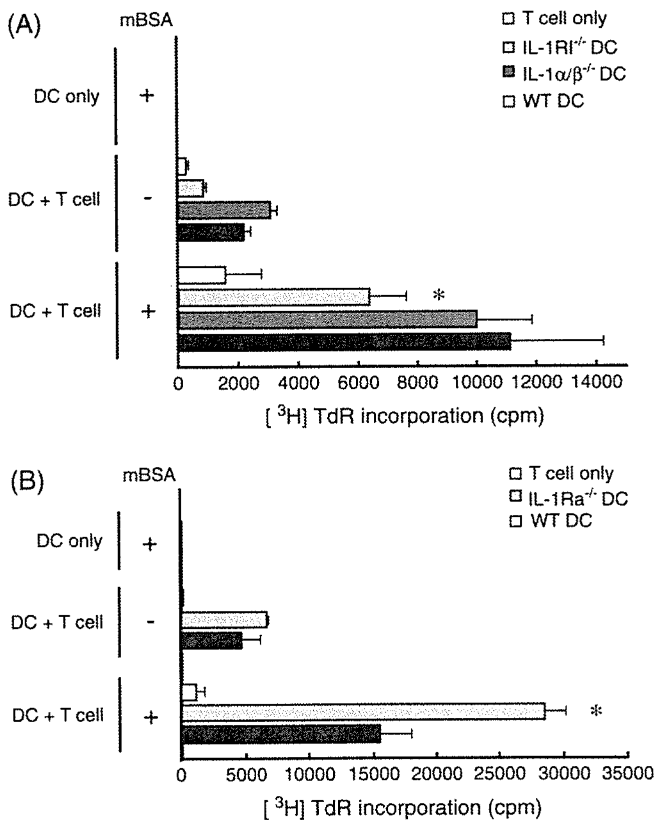


**Fig. 6.** Effects of DC-derived IL-1 on CD4$^+$ T cell proliferation. CD4$^+$ T cells (1 × 10$^5$ per well) from mBSA-immunized wild-type mice were co-cultured with CD11c$^+$ DC cells from a different genotype (1 × 10$^4$ per well) in the presence or absence of mBSA. The proliferative responses of T cells were measured by [$^3$H]TdR incorporation. (A) Wild type (WT), IL-1α/β$^{-/-}$ and IL-1RI$^{-/-}$ DCs. (B) WT and IL-1Ra$^{-/-}$ DCs. [$^3$H]TdR incorporation was measured in three wells; the mean ± SD of these values is shown. Similar results were obtained in three independent experiments. *$P < 0.05$ versus wild type.

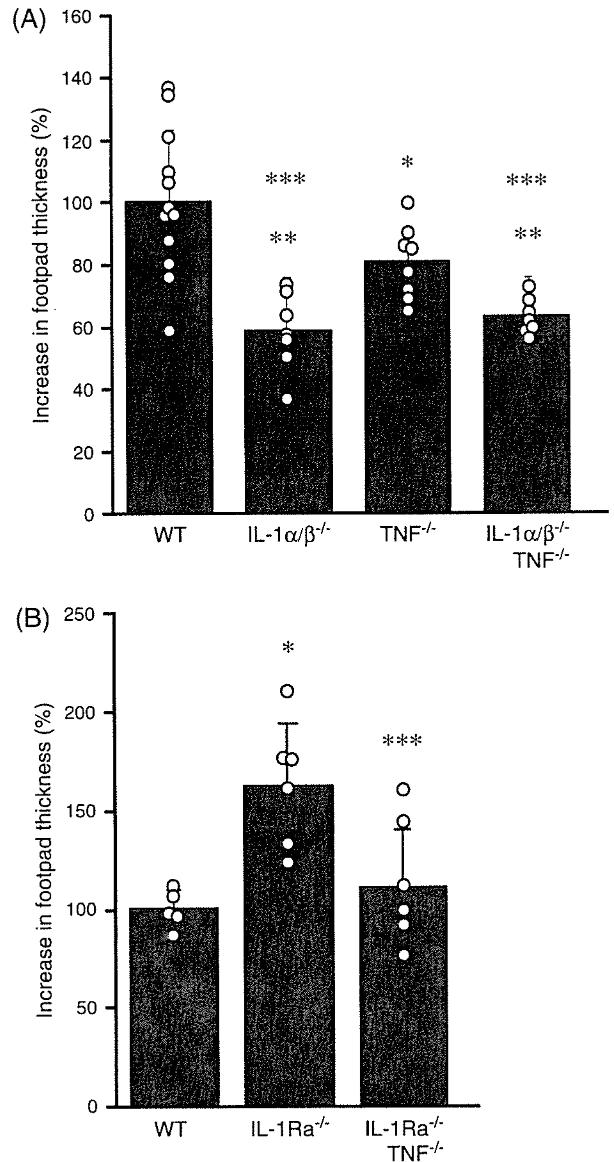


**Fig. 7.** Roles of IL-1 and TNF in DTH responses. mBSA-induced DTH responses in wild-type, IL-1α/β$^{-/-}$, TNF$^{-/-}$ and IL-1α/β$^{-/-}$TNF$^{-/-}$ mice (A) and in wild-type, IL-1Ra$^{-/-}$ and IL-1Ra$^{-/-}$TNF$^{-/-}$ mice (B) were examined as in Fig. 1. Circles indicate disease severity of individual mice. The mean ± SD of each group is shown. (A) Wild type, $n = 12$; IL-1α/β$^{-/-}$, $n = 7$; TNF$^{-/-}$, $n = 8$ and IL-1α/β$^{-/-}$TNF$^{-/-}$, $n = 7$. **$P < 0.01$, *$P < 0.05$ versus wild-type mice and # $P < 0.05$ versus TNF$^{-/-}$ mice. (B) Wild type, $n = 5$; IL-1Ra$^{-/-}$, $n = 6$ and IL-1Ra$^{-/-}$TNF$^{-/-}$, $n = 6$. *$P < 0.05$ versus wild-type mice and ***$P < 0.05$ versus IL-1Ra$^{-/-}$ mice.

induced downstream of IL-1 signaling and a portion of the biological activities induced by IL-1 may occur through the induction of TNF.

## Discussion

Using IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice, we demonstrated that IL-1β, more so than IL-1α, plays an important role in the development of DTH responses. The proliferative recall responses of T cells derived from both IL-1$\alpha/\beta^{-/-}$ mice and IL-1$\beta^{-/-}$ mice, but not those from IL-1$\alpha^{-/-}$ mice, were significantly reduced, suggesting that IL-1β functions in T cell sensitization. With regard to the function of IL-1 in the sensitization of T cells, we previously showed that IL-1 activate T cells by inducing CD40L and OX40 expression in T cells (26). Recently, it was also shown that IL-1β signaling can activate naive T cells through activation of c-Rel (27).

The involvement of IL-1β in T cell sensitization was also suggested by the responses observed during airway hypersensitivity responses (28, 29). In contrast, IL-1α, but not IL-1β, is crucial for the CHS reaction (19), and both IL-1α and IL-1β are important for the antibody production against mBSA during DTH reaction. As IL-1α and IL-1β bind the same receptors, it seems likely that differential expression of these molecules may explain the differential roles of these molecules among different allergic reactions. In support for this idea, we showed that IL-1β is mainly produced during DTH reactions. The reason why specific IL-1 species are produced during different allergic reactions is not resolved completely. With regard to this, it was reported that different APCs, the major producer of IL-1 during T cell sensitization (30), are required for the antigen presentation during sensitization phase; DCs play a key role in DTH reactions while Langerhans cells (LCs) play an essential role in CHS reactions (3). Human DC1 cells, derived from monocytes, synthesize IL-1α but DC2 cells, derived from plasmacytoid cells, do not (31). $CD11a^-CC81^-MyD\text{-}1^+$ DCs from bovine afferent lymph synthesized IL-1α and stimulated both $CD4^+$ and $CD8^+$ T cells, while $CD11a^+CC81^+MyD\text{-}1^-$ DCs that did not synthesize IL-1α could not stimulate $CD8^+$ T cells (32). On the other hand, both IL-1α and IL-1β are produced in LCs (19). Thus, it is possible that $CD4^+$ T cells that are involved in the DTH response as well as in airway hypersensitivity are stimulated by a particular subpopulation of DCs that synthesizes only IL-1β. However, the exact mechanism for the functional discrimination between IL-1α and IL-1β remains to be elucidated.

DTH response has been reported to occur normally in IL-1$\beta^{-/-}$ mice (18). The apparent discrepancy to our results, following two points should be noted. First, the mBSA dosage and the immunization route that they used were different from that we used in the present experiments. It is possible that other cytokines may compensate the deficiency of IL-1β when antigenic stimulation is very strong. In support for this notion, Shornick *et al.* (33) demonstrated that IL-1β deficiency could be overcome either by application of very high doses of sensitizing antigen or by local intra-dermal injection of recombinant IL-1β before antigen application. Second, the genetic backgrounds of the mice used in the experiments were different between these two studies; we used IL-1$\beta^{-/-}$ mice that were backcrossed to either C57BL/6J mice or BALB/cA mice for eight generations, whereas they studied in 129 × B6 IL-1$\beta^{-/-}$ mice. It is possible that a small difference of footpad swelling could not be detected on the mixed background mice.

IFN-γ is a $T_h1$ cytokine and is critically involved in the development of DTH responses (34, 35). IFN-γ production by $CD4^+$ T cells was reduced both in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice after immunization with mBSA, suggesting that IL-1 induces the production of IFN-γ either by activating T cells or, in the case of IL-1α, by directly stimulating IFN-γ gene transcription as a transcription factor (36). This suppression of IFN-γ production, however, does not explain the differential sensitivity of IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice to the DTH responses, because the IFN-γ production was similar between both mutant mice. Thus, IFN-γ production by IL-1$\alpha^{-/-}$ T cells is sufficient or some other factors compensate for the deficiency of this cytokine to induce a full magnitude DTH response. With this respect, it was reported that DTH reaction is suppressed in IFN-$\gamma^{-/-}$ mice when mice were immunized with antigen alone (37, 38). Since DTH response and pro-inflammatory cytokine expression are much enhanced in the presence of adjuvant, our results suggest that some other cytokines may compensate for the function of IFN-γ. We observed similar compensatory effects of adjuvant for the deficiency of a cytokine in airway hypersensitivity responses (26). In this connection, we examined expression of chemokines in IL-1-deficient mice. The expression levels of RANTES, IP-10 and monocyte chemoattractant protein-1 (MCP-1) were similar between IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice in the elicitation phase. Thus, these chemokine expression levels also could not explain the difference of the DTH responses between IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice, indicating involvement of other factors.

We also discovered that antibody production against mBSA was reduced both in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice, indicating that both IL-1α and IL-1β are involved in this humoral immune response. However, T cell sensitization was reduced only in IL-1$\beta^{-/-}$ mice. Thus, IL-1 may also function in the process of antibody production other than T cell sensitization. With regard to this, it is known that IL-1 activates DCs and induces maturation of DCs (39–42). IL-1 also stimulates the proliferation of splenic B cells after crossing of their surface Igs (43) and by signals through CD40 and IL-4R (44), and promotes the survival of germinal center B cells (45). IL-1 is strongly expressed in follicular DCs in the germinal center, which play important roles in affinity maturation and isotype switch of Igs through interaction with B cells (46). As another possibility, since IFN-γ plays an important role in the class switching of IgG in B cells (47, 48), the reduction of IFN-γ production in IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice may cause reduction of antibody production against mBSA. These data also indicate that antibody levels against mBSA do not affect greatly the development of DTH, as normal DTH responses were observed in IL-1$\alpha^{-/-}$ mice. The relative independence of DTH on humoral immune responses was also suggested by Lagrange *et al.* (49).

In addition to a role in the sensitization of T cells, IL-1 is also involved in the elicitation of local inflammation during DTH responses. The development of DTH was severely suppressed following the adoptive transfer of mBSA-immunized $CD4^+$ T cells into IL-1RI$^{-/-}$ mice, but not IL-1$\alpha/\beta^{-/-}$ mice,

suggesting that activated CD4$^+$ T cell-derived IL-1 plays an important role in the elicitation phase. We also demonstrated that IL-1β derived from activated memory T cells is required for the activation of DCs that activate antigen-specific T cells at inflammatory sites. It is known that IL-1 enhances T-dependent immune responses by amplifying the function of DCs (50). IL-1β acts on APCs to enhance the *in vivo* proliferation of antigen-stimulated naive CD4$^+$ T cells (40), but it does not induce the expression of CD80, CD134 ligand, 4-1BBL or glucocorticoid-induced TNF (40). IL-1β induces functional maturation of DCs by inducing CD40, CD86 and MHC class II (39), although we could not detect any defects in IL-1-deficient DCs (30). In addition, IL-1β induces DCs to secrete IL-12 leading to the activation of cellular immunity (42). Thus, T cell-derived IL-1β may enhance maturation of immature DCs and activate DCs to enhance inflammation during the elicitation phase of the DTH response. These observations suggest dual functions for IL-1 in DTH reactions. During the sensitization phase, IL-1 produced by DCs activates T cells; during the elicitation phase, T cell-derived IL-1 activates DCs to enhance antigen presentation and/or cytokine production.

As IL-1 deficiency only partially reduced the DTH response, it was suggested that other factors contributed to the response. Accordingly, we examined the contribution of TNF, which plays an important role in the development of inflammation during the elicitation phase (26), and found that the elevated DTH response in IL-1Ra$^{-/-}$ mice was cancelled by TNF deficiency, indicating that the actions of IL-1 are mediated in part by TNF. Other hypersensitivity responses, collagen- and antigen-induced arthritis and experimental autoimmune encephalomyelitis, are also suppressed in TNF$^{-/-}$, TNFRI$^{-/-}$ and TNFRII$^{-/-}$ mice, indicating the importance of this cytokine (51–56). Interestingly, however, the severity of DTH in IL-1α/β$^{-/-}$TNF$^{-/-}$ triple mutant mice did not differ from IL-1$^{-/-}$ mice (Fig. 7). With regard to this, we previously demonstrated that TNF production is induced downstream of IL-1 signaling and TNF is not induced in the absence of IL-1 (26). Thus, it seems unlikely that TNFs compensate for the deficiency of IL-1. In this context, several factors are suggested to be involved in the development of DTH responses. Exacerbated inflammation is observed when MCP-1 (57, 58), IL-12 and IL-18 (1, 59) are over-expressed, and on the contrary, DTH responses are suppressed in the absence of these molecules, suggesting that these molecules contribute to the DTH responses through IL-1-independent pathway. Recently, it was also demonstrated that IL-16, a chemoattractant for CD4$^+$ leukocytes (60), is induced in the inflamed footpads and the treatment with anti-IL-16 can inhibit the recruitment of not only CD4$^+$ T cells but also macrophages (24).

In summary, IL-1β, more so than IL-1α, is critical in the development of DTH responses, functioning as both a T cell co-stimulatory molecule and a pro-inflammatory cytokine. T cell-derived IL-1 contributes to the induction of local inflammation by activating DCs. Polymorphism in the genes for both IL-1β and IL-1Ra has been shown to influence both DTH responses against *Mycobacterium tuberculosis* and disease manifestations in human tuberculosis (61). Therefore, our findings may provide a rationale explaining the dependence of the disease on IL-1 gene polymorphisms, which may aid in the future development of novel therapeutics.

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**Abbreviations**

| | |
|---|---|
| AP | alkaline phosphatase |
| APC | antigen-presenting cell |
| CHS | contact hypersensitivity |
| DC | dendritic cell |
| DTH | delayed-type hypersensitivity |
| HPRT | hypoxanthine phosphoribosyltransferase |
| IL-1Ra | IL-1R antagonist |
| IL-1RI | IL-1R type I |
| IL-1RII | IL-1R type II |
| LC | Langerhans cells |
| LN | lymph node |
| mBSA | methyl BSA |
| MCP-1 | monocyte chemoattractant protein-1 |
| RT | reverse transcription |
| r.t. | room temperature |
| s.c. | subcutaneously |
| TNF | tumor necrosis factor |

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