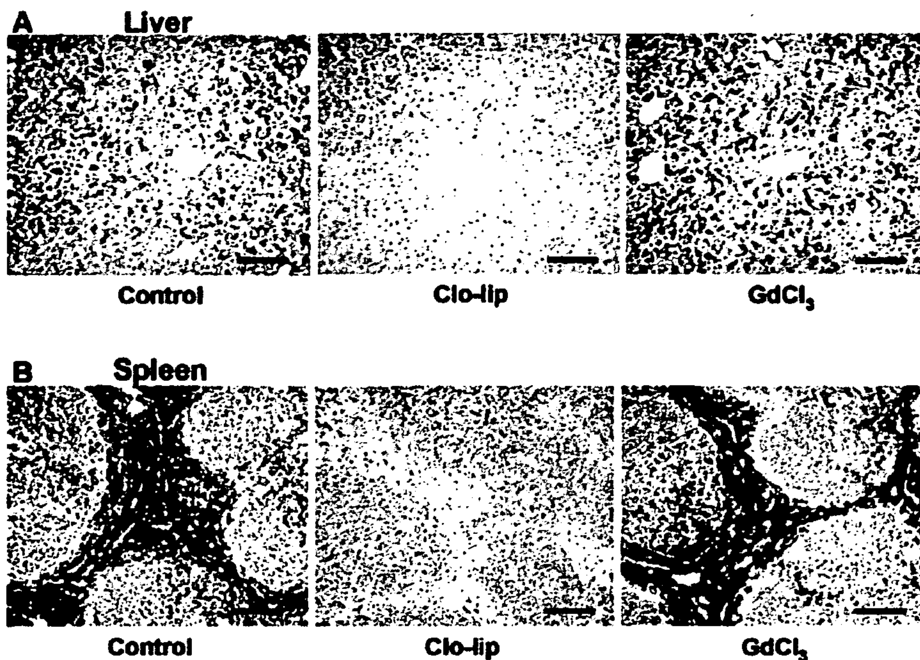


Fig. 1. Effect of Clo-lip and $GdCl_3$ on the depletion of macrophages in mice. Saline (Control) or Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.v. in C57BL/6 mice on Days 0, 3, and 5, and the livers and spleens were taken on Day 8. $GdCl_3$ (10 mg/kg) was administered i.v. in C57BL/6 mice on Days 0, 4, and 7, and the livers and spleen were taken on Day 9. The liver (A) and spleen (B) were subjected to immunostaining with F4/80 antibody and visualized using diaminobenzidine (brown). The results are representative of three mice. Original scale bars, 200 μm.

results indicate that Clo-lip efficiently eliminates phagocytic macrophages in mice; however, it remains possible that $GdCl_3$ inhibits the functions of macrophages, even though it did not eliminate them [24]. Therefore, we used Clo-lip and $GdCl_3$ in the next experiments.

Serum IL-18 levels were increased markedly by treatment with *P. acnes* and LPS, whereas the administration of Clo-lip, three times (1 day before and 2 and 4 days after *P. acnes* injection), or $GdCl_3$, three times (2 days before and 3 and 5 days after *P. acnes* injection), showed no obvious effect on the serum IL-18 levels (**Fig. 2A**). Consistent with this, Western blot analysis showed that an 18-kDa, mature form of IL-18 was detected in the sera of *P. acnes* and LPS-treated mice and that the band was unchanged by Clo-lip or $GdCl_3$ treatment (Fig. 2B). These results indicate that activated macrophages are not critically involved in the induction of serum IL-18 in mice treated with *P. acnes* and LPS and suggest that other cells are the source of serum IL-18.

As Clo-lip and $GdCl_3$ showed the same effect on the induction of serum IL-18 levels, and Clo-lip efficiently eliminated F4/80$^+$ macrophages in mice, we used Clo-lip in the following experiments.

IL-18 levels in various organs in mice

We then examined the IL-18 levels in various organs in mice. In untreated, control mice, the IL-18 levels were marginal in the liver, lung, and spleen and more pronounced in the intestines (duodenum, jejunum, ileum, and colon), especially so in the duodenum (**Fig. 3A**). Treatment with *P. acnes* alone induced IL-18 more than twofold in each organ except for the lung and colon. Treatment with *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased them slightly in the duodenum, jejunum, and ileum, compared with those with *P. acnes* alone.

Western blot analysis showed that proIL-18 was expressed at a low amount in the livers of untreated mice, and *P. acnes* treatment induced proIL-18 and mature IL-18 in the liver (Fig. 3B). Treatment with *P. acnes* and LPS resulted in a further increase of both forms of IL-18. The spleens of untreated mice expressed proIL-18 and a band lower than 18 kDa, suggesting that mature IL-18 is not expressed in the untreated spleen. The expression of proIL-18 and mature IL-18 was induced by *P. acnes* alone and increased further by *P. acnes* and LPS in the spleen. In contrast, both forms existed already in the untreated duodenum, and mature IL-18 was increased markedly in the duodenum by *P. acnes* alone and by *P. acnes* and LPS. In the jejunum and ileum, mature IL-18 was mainly expressed, and the expression was increased and decreased slightly by *P. acnes* alone and *P. acnes* and LPS, respectively, compared with the untreated control. In the colon, the expression of pro-IL-18 was higher than that of mature IL-18 in the untreated control, and *P. acnes* treatment reduced pro-IL-18 and increased mature IL-18 expressions. The expression of proIL-18 was increased by *P. acnes* and LPS, compared with *P. acnes* alone. These results indicate that *P. acnes* priming in mice leads to the accumulation of mature IL-18 in these organs and renders the mice susceptible to LPS, that LPS challenge induced the release of mature IL-18, and that the diverse expression of pro- and mature IL-18 occurs in each organ and even in the intestines.

Effect of macrophage depletion on the IL-18 levels in organs in mice

As Figure 2 illustrates that Clo-lip showed no effect on serum IL-18 levels, the effect of Clo-lip on the IL-18 levels in various organs in mice was then examined. Treatment of mice with *P. acnes* and LPS markedly increased the IL-18 levels in the liver and spleen but not in the lung, and the administration of Clo-lip, three times (1 day before and 2 and 4 days after *P.*

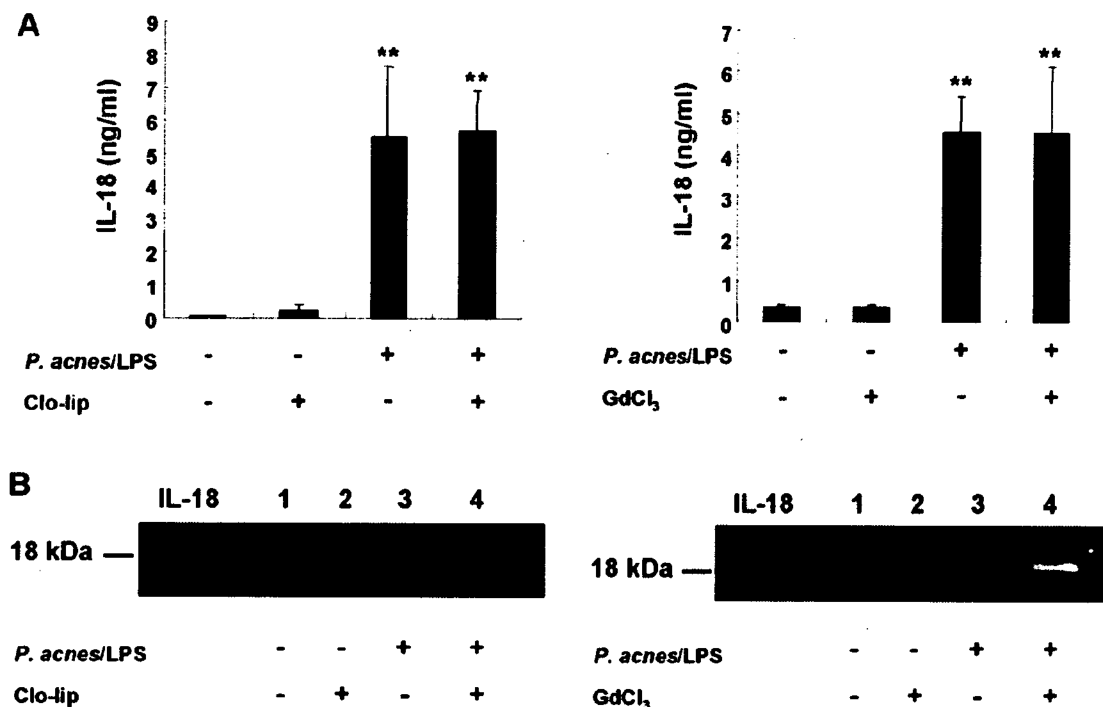


Fig. 2. Effect of Clo-lip and $GdCl_3$ on the induction of serum IL-18 in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 μg/mouse) or PBS. Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administerted i.p. into the mice 1 day before and 2 and 4 days after *P. acnes* administration. $GdCl_3$ (10 mg/kg) was administerted i.v. into the mice 2 days before and 2 and 5 days after *P. acnes* administration. Blood was then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the sera were determined by ELISA. The results were expressed as the means ± SD for five mice. **, $P < 0.01$, compared with untreated mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. mrIL-18 (1 ng) was loaded as a control. The results are representative of five mice of each group.

acnes injection) in the mice, slightly, but not significantly, decreased in the IL-18 levels in the liver (**Fig. 4A**). However, a substantial amount of IL-18 remained in the liver following the Clo-lip treatment. In addition, the IL-18 levels in the spleens of mice treated with *P. acnes* and LPS did not change following the Clo-lip treatment. The IL-18 levels in the intestines are also unchanged by the Clo-lip treatment (data not shown). Consistent with this, Western blot analysis showed that the expression of mature IL-18 was almost unchanged in the livers and spleen of mice treated with *P. acnes* and LPS after the Clo-lip administration (Fig. 4B). These results further indicate that IL-18 expressed in activated macrophages in organs and tissues does not cause the elevation of serum IL-18 levels.

Effect of macrophage depletion on LPS-induced liver injury in *P. acnes*-primed mice

As it has been reported that treatment with *P. acnes* and LPS induces IL-18-dependent, acute liver injury in mice through the induction of hepatotoxic factors such as TNF-α and that Kupffer cells are the major source of IL-18 in the liver [28–30], we next examined the effect of Clo-lip on the liver injury. The levels of the liver enzymes, AST and ALT, in sera were increased markedly by treatment with *P. acnes* and LPS, and their levels were decreased significantly by the three-time administration of Clo-lip (**Fig. 5A**). However, the serum AST and ALT levels were still high compared with those of untreated mice. Treatment with *P. acnes* and LPS resulted in a marked increase in serum TNF-α levels, and the induction of serum TNF-α was slightly, but not significantly, reduced by Clo-lip treatment (Fig. 5B). Consistent with this, histological analysis showed that treatment with *P. acnes* and LPS induced severe liver injury (necrotic change and granuloma formation) and cell infiltration, and treatment with *P. acnes* alone also induced granuloma formation and cell infiltration (**Fig. 6**). No granuloma was observed, and the cell infiltration was reduced by Clo-lip administration. However, the cell infiltration and necrotic change were still observed in the Clo-lip-administered liver. These results suggest that Kupffer cells are actually involved in liver injury in mice treated with *P. acnes* and LPS and that IL-18 and TNF-α from non-Kupffer cells also contribute to the liver injury.

DISCUSSION

IL-18 was identified originally as a potent IFN-γ-inducing factor in the serum and livers of mice, which had been administered *P. acnes* and LPS sequentially [1]. IL-18 was first identified in activated macrophages, such as Kupffer cells, in the liver. Further investigations have revealed that IL-18 is also expressed in nonimmune cells, such as keratinocytes, osteoblasts, adrenal cortex cells, epithelial cells of various organs and tissues, microglial cells, and synovial fibroblasts [2–4]. Increased levels of IL-18 have been reported in the sera

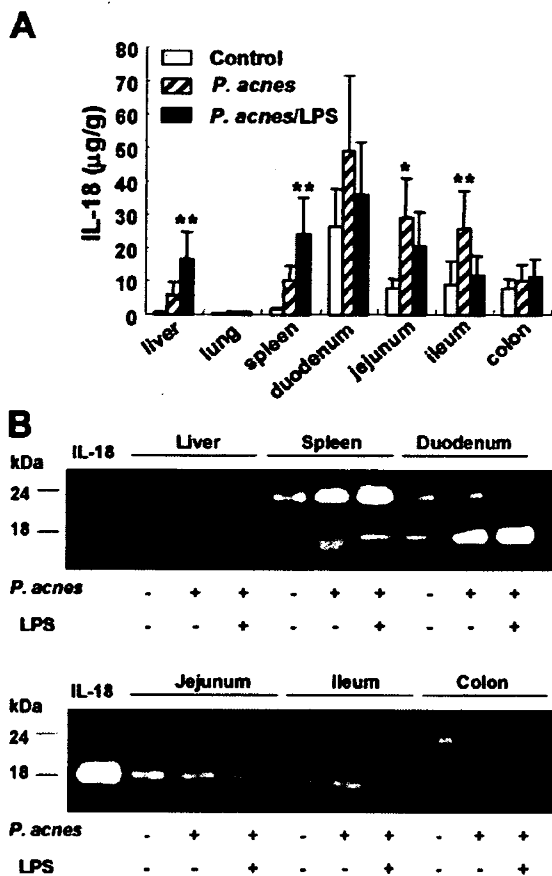


Fig. 3. IL-18 and levels in various organs or tissues in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 µg/mouse) or PBS. Organs or tissues were then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the samples were determined by ELISA. The results were expressed as the means ± SD for five mice. *, $P < 0.05$, and **, $P < 0.01$, compared with untreated mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. mrIL-18 (10 ng) was loaded as a control. The results are representative of five mice of each group.

from patients with a wide variety of diseases, including autoimmune and inflammatory disorders [6–11], allergy [12], allograft rejection [13], and infectious diseases [14–16], and the elevated serum IL-18 levels are considered to be a parameter for the disease severity and a diagnostic marker. However, it is unclear whether the major source of serum IL-18 is immune cells, such as activated macrophages, or nonimmune cells, such as epithelial origin. The present study used Clo-lip to deplete phagocytic macrophages selectively in mice. After the ingestion of Clo-lip into macrophages, phospholipases in the lysosomes degrade the phospholipid bilayers, releasing the clodronate into the cells, resulting in cell death, and the i.v. injection of Clo-lip into mice results in a selective depletion of macrophages, including liver and spleen macrophages [21, 22]. This study demonstrated that the three-time administration of Clo-lip eliminated F4/80$^+$ macrophages in the liver and spleen (Fig. 1), suggesting that the Clo-lip treatment depletes phagocytic macrophages in mice and that phagocytic macrophages are not critically involved in the increase in serum IL-18 levels. The present study also used $GdCl_3$ to inactivate phagocytic macrophages, as it is reported that the i.v. injection of $GdCl_3$ not only blocks phagocytosis but also eliminates these cells [24, 25]; however, we were unable to deplete F4/80$^+$ macrophages in the liver and spleen by the i.v. injection of $GdCl_3$ three times (Fig. 1), and the administration of $GdCl_3$ three times showed no obvious effect on serum IL-18 levels, which were comparable with Clo-lip (Fig. 2). It is also reported that $GdCl_3$ does not significantly reduce the number of phagocytically active cells in the liver [31], that splenic macrophages are less vulnerable to $GdCl_3$ [25], and that $GdCl_3$ treatment results in a significant increase in serum levels of TNF-α, IL-6, and liver enzymes, ALT and AST [32]. Therefore, we consider that Clo-lip is more effective in the inactivation and elimination of phagocytic macrophages than $GdCl_3$, and we used Clo-lip mainly.

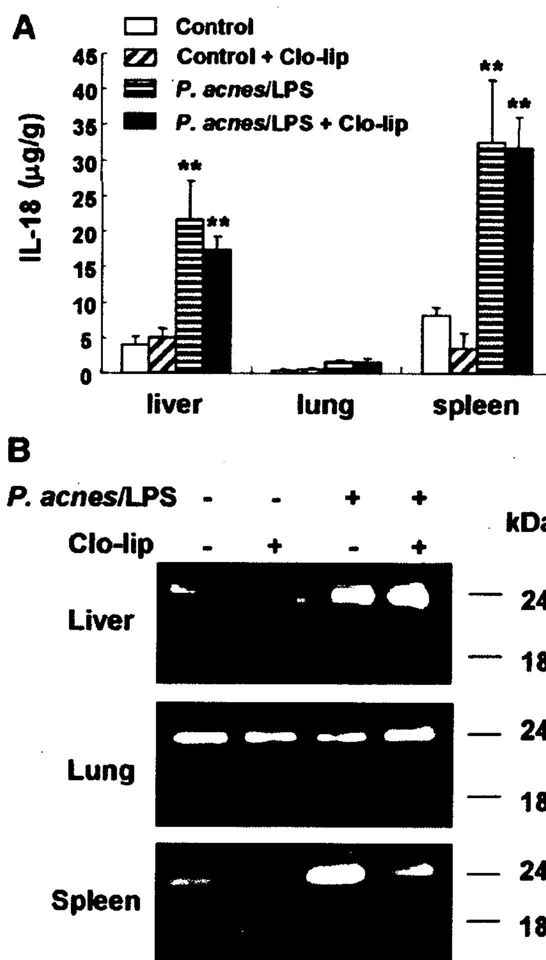
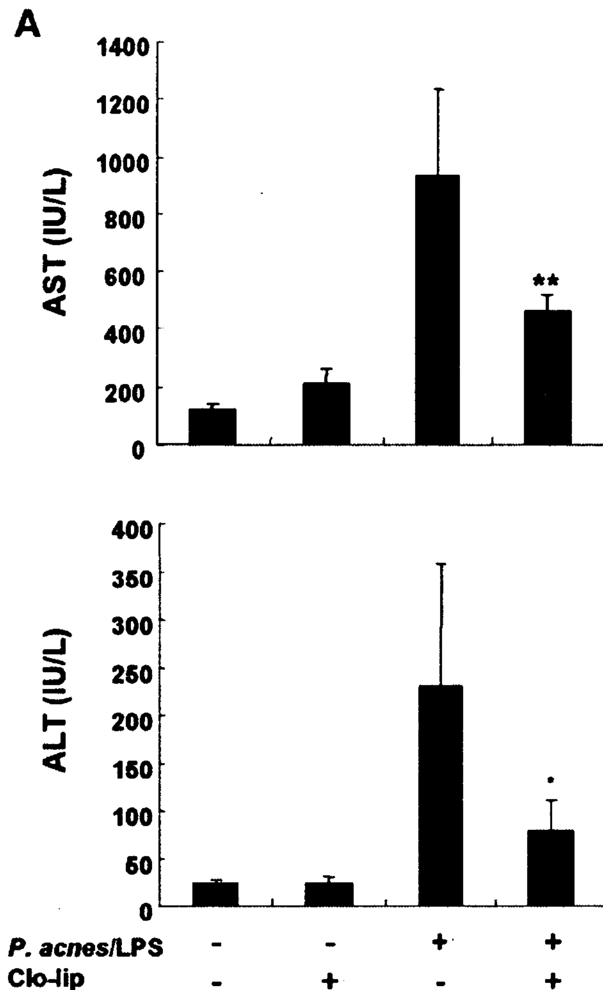


Fig. 4. Effect of macrophage depletion on the IL-18 levels in the liver, lung, and spleen in mice. (A) *P. acnes* (1 mg dry weight/mouse) or PBS was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with LPS (1 µg/mouse) or PBS. Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* administration. The liver, lung, and spleen were then taken from the mice 2 h after LPS challenge, and the levels of IL-18 in the samples were determined by ELISA. The results are expressed as the means ± SD for five mice. **, $P < 0.01$, compared with untreated (Control) mice. (B) Samples in A were subjected to Western blotting using anti-mouse IL-18 pAb. The results are representative of five mice of each group.



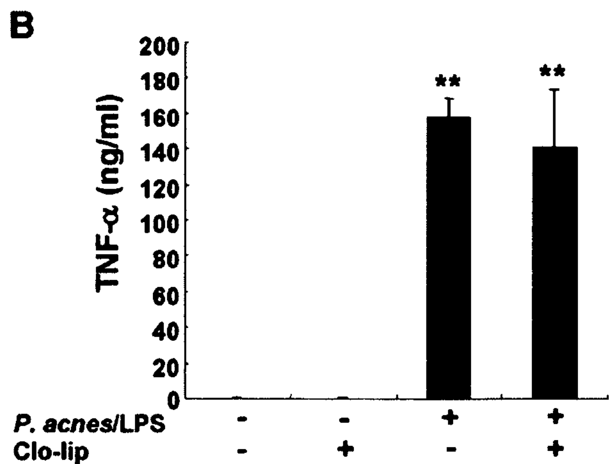


Fig. 5. The effect of Clo-lip administration on serum levels of liver enzymes and TNF-α. (A) PBS or *P. acnes* (1 mg dry weight/mouse) was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v. with PBS or LPS (1 μg/mouse). Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* priming. Blood was taken from the mice 2 h after LPS or PBS challenge, and the serum AST and ALT levels were measured. (B) The levels of TNF-α in the samples in A were determined by ELISA. The results are expressed as the means ± SD for five mice. *, $P < 0.05$, and **, $P < 0.01$, compared with *P. acnes* and LPS.

Analysis using IFN-γ-deficient mice showed that the induction of LPS hypersensitivity by *P. acnes* priming is mediated strictly by IFN-γ [33, 34]. IL-18 with IL-12 strongly induces IFN-γ from activated $CD4^+$ Th1 cells and NK cells [1], and IFN-γ also in turn can regulate the secretion of bioactive IL-18, constituting a feedback loop between these cytokines [33]. IFN-γ plays a major role in immune regulation. It activates various macrophage functions, including cytokine production, antimicrobial activity, and antigen processing and presentation [35]. In addition, IFN-γ activates nonimmune cells, including keratinocytes, epithelial cells, and fibroblasts [36–38]. IFN-γ-activated keratinocytes express a number of chemokines, cytokines, and adhesion molecules [36, 37]. The priming of oral epithelial cells and gingival fibroblasts with IFN-γ induces sensitivity to pathogen-associated molecular patterns, including LPS and peptidoglycans [38, 39]. Therefore, the induction of IFN-γ during *P. acnes* priming in mice is critically involved in the accumulation of a mature IL-18 from each organ.

The IL-18 levels were increased in the liver and spleen but decreased in the intestines by *P. acnes* and LPS compared with *P. acnes* alone (Fig. 3A). Immunoblot analysis showed that proIL-18 was detected mainly in the liver and spleen in untreated mice (Figs. 3B and 4B), whereas the diverse expression of proIL-18 and mature IL-18 was observed in the intestines of mice (Fig. 3B). Furthermore, treatment with *P. acnes* and LPS induced a marked increase in IL-18 levels in the liver and spleen but decreased them slightly in the intestines compared with those with *P. acnes* alone. Therefore, it is possible that the mechanism of accumulation and release of IL-18 are different depending on the organ and even in the intestines.

F4/80 is expressed on most resident tissue macrophages, including the red pulp macrophages in the spleen, Kupffer cells in the liver, and Langerhans cells in the skin [40]. This study showed that the Clo-lip treatment did not reduce the IL-18 levels in the livers and spleens of mice treated with *P. acnes* and LPS (Fig. 4). These findings suggest that the tissues or cells other than activated macrophages also express IL-18 in the liver and spleen. DC are able to produce IL-18 [41] and are not phagocytic [42], and the phenotypes of hepatic DC are $F4/80^{low}$ or $F4/80^-$ [43]. A recent study revealed that DC in the liver play important roles in the induction and regulation of immune responses [43]. Therefore, it is possible that the administration of Clo-lip did not efficiently eliminate the IL-18-expressing DC in the liver. This possibility may be the case in the spleen, as there is no report that T and B cells produce IL-18. However, murine bone marrow-derived DC produce IL-18 at ~100 pg/ml levels for 7 or 8 days of culture [41], whereas serum IL-18 levels in mice treated with *P. acnes* and

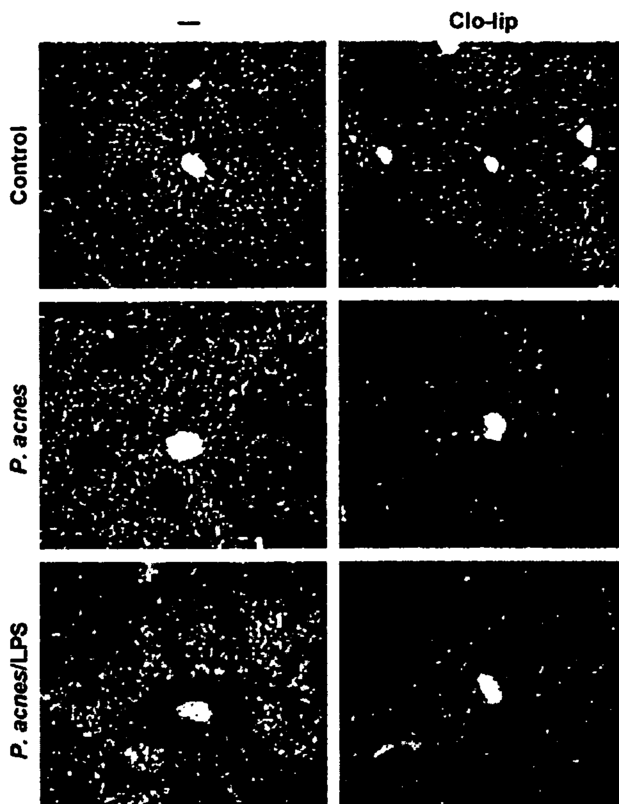


Fig. 6. Effect of Clo-lip on LPS-induced liver injury in *P. acnes*-primed mice. PBS or *P. acnes* (1 mg dry weight/mouse) was administered i.p. to C57BL/6 mice, and 7 days later, they were challenged i.v., with PBS or LPS (1 μg/mouse). Clo-lip (fivefold-diluted, 0.2 ml/mouse) was administered i.p. to the mice 1 day before and 2 and 4 days after *P. acnes* priming. Liver specimens were then sampled 2 h after PBS or LPS challenge, and liver tissue sections were stained with H&E. The results are representative of five mice of each group. Original scale bars, 200 μm.

LPS were ng/ml levels (Fig. 2). Therefore, it is conceivable that the contribution of CD-derived IL-18 to serum IL-18 levels is marginal. Another possible source in the liver is oval cells, which are proliferating, epithelial cells with an ovoid nucleus and appear in liver generation, and IL-18 was expressed in oval cells in the regenerating liver at mRNA and protein levels [44]. Therefore, it is also possible that pathological changes caused by *P. acnes* induce IL-18-expressing oval cells in the liver. NK cells or NK T cells may also be the source of IL-18. Further studies are required to clarify these points.

It has been reported that treatment with *P. acnes* and LPS induces IL-18-dependent liver injury through the induction of TNF-α [28–30] and suggested that Fas/Fas ligand-mediated IL-18 secretion from Kupffer cells causes the liver injury in mice [29]. Analysis using IL-18 transgenic mice showed that IL-18 plays a key role in regulating hepatocyte apoptosis in vivo [45], indicating that IL-18 is critically involved in the liver injury. This study showed that depletion of F4/80$^+$ macrophages by Clo-lip in *P. acnes* and LPS-treated mice reduced serum AST, ALT, and TNF-α levels and pathological change of the liver (Figs. 5 and 6). These results suggest that Kupffer cells play an important role for the onset of liver injury. However, a substantial amount of the liver enzymes and the liver injury was found in the Clo-lip-treated mice, suggesting that IL-18 and TNF-α from liver tissues or cells other than Kupffer cells also cause the liver injury. It is also possible that IL-18 in circulation is involved in the liver injury, as the i.p. injection of rIL-18 is able to induce the liver injury in *P. acnes*-primed mice [29].

In conclusion, the present study suggests that serum IL-18 is derived from nonphagocytic macrophages, probably from epithelial cells of various organs and tissues in mice treated with *P. acnes* and LPS. Increased levels of serum IL-18 are associated with a wide variety of diseases [6–16]; therefore, it is possible that the serum IL-18 is derived from diseased organs or tissues. IL-18 is not only an important regulator of innate and acquired immune responses but also a potent, proinflammatory cytokine, which regulates a wide variety of autoimmune and inflammatory diseases [2–4]. Bone malformation and the exacerbation of colitis were reported in IL-18 transgenic mice [46, 47], and the overexpression of IL-18 with the Keratin 5 promoter in mice showed exacerbated and prolonged, allergic and nonallergic, inflammatory skin reactions [48]. These findings indicate that the overexpression of IL-18 results in deleterious alterations in the organs and tissues. Therefore, IL-18 overexpressed in these organs and tissues may be an important, therapeutic target for the treatment of diseases.

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Oral bacterial lipopolysaccharide acts in mice to promote sensitisation to ovalbumin and to augment anaphylaxis via platelets

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ABSTRACT

Microbial infection is thought to modulate allergic disorders, and we previously demonstrated that not only mast cells (which release histamine), but also platelets are involved in the anaphylaxis induced in mice sensitised to ovalbumin (OVA). Here, we examined the effects of a lipopolysaccharide (LPS) from the oral bacterium *Prevotella intermedia* (*Pi*) on OVA-induced anaphylaxis. Upon intraperitoneal co-injection of *Pi*-LPS plus OVA into BALB/c mice, the *Pi*-LPS displayed a potent adjuvant effect comparable to that of alum (a standard adjuvant) in terms of its abilities to induce both anaphylactic shock and histamine-release following an antigen (OVA)-challenge. Moreover, an injection of *Pi*-LPS given to OVA+alum-sensitised mice shortly before an OVA-challenge augmented the shock-response. This LPS-pretreatment did not affect histamine-release, but did augment pulmonary platelet accumulation. Histamine was not by itself causal for shock-induction in sensitised mice. These results suggest that oral bacteria and/or their constituents (such as LPS) may help to sensitise the host to an antigen or exacerbate the host's allergic reactions ("aggravation effect"), probably by enhancing the platelet response to the antigen OVA.

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1. Introduction

Environmental factors are thought to be important in the development of allergic disorders. Indeed, infection or bacterial components (such as lipopolysaccharide (LPS), DNA, and superantigens) have been reported to exert bidirectional influences (protection or exacerbation) on both the establishment and development of allergic disorders.[1–3] Black-pigmented bacteria, such as *Porphyromonas gingivalis* and *Prevotella intermedia* (*Pi*), are the dominant gram-negative bacteria in the periodontal pockets of patients with periodontitis.[4] Indeed, these bacteria, which are also prevalent in endodontal and periapical infections,[5,6] are believed to contribute to the development of periodontitis and osteomyelitis.[7] Herzberg

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and Meyer emphasized that through daily oral hygiene procedures or dental surgical procedures, exposure to oral microorganisms may occur repeatedly and/or frequently in patients with periodontitis.[8]

Interestingly: (a) oral administration of LPS in mice increases its level in the blood and results in an exacerbation of collagen-induced arthritis;[9] (b) transgenic mice with an autoantibody against red blood cells develop anemia only when bred in a conventional environment, not when bred under germ-free conditions;[10] and (c) intragingival injection into mice of small doses of Pi-LPS induces the histamine-forming enzyme, histidine decarboxylase, in extraoral tissues (such as liver, lung, and spleen).[11] These findings suggest that LPS from oral bacteria may, in various ways, influence the development of allergic disorders.

We previously found that platelets are involved in ovalbumin (OVA)-induced anaphylaxis in mice, including mast cell-deficient mice.[12] In the present study, we examined the effects of Pi-LPS on sensitisation, induction of anaphylactic shock, histamine (H) release, and platelet responses in a murine OVA-induced anaphylaxis model.

2. Materials and methods

2.1. *Animals and materials*

BALB/c female mice were used in the present experiments. LPS from *Prevotella intermedia* ATCC 25611 (abbreviated as Pi) was prepared by the method of Galanos et al.[13] (phenol–chloroform–petroleum ether extraction).[14,15] The purity of this LPS is 5.2 μg protein/mg of preparation.[16] OVA (from chickens; 5× crystallized) was purchased from Seikagku Corp (Tokyo, Japan). All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

2.2. *Immunization and provocation of anaphylaxis*

A suspension (0.5 ml) containing OVA (50 μg) alone, alum (3 mg) + OVA, or LPS (at the doses indicated in each experiment) + OVA was injected intraperitoneally (i.p.) on day 0 and again on day 10. The first injection was given to mice at 6 weeks of age. The antigen-challenge was delivered on days 20–25 by giving an intravenous (i.v.) injection of OVA dissolved in saline (0.25 ml per mouse via a tail vein) at the dose indicated in each experiment.

2.3. *Scoring of anaphylactic shock*

The highest score given to the severity of anaphylactic shock within 10 min of the OVA-challenge was recorded for each mouse. The scoring was as follows: 0 (no shock signs), 1 (staggering), 2 (crawling and prostration), and 3 (prostration and weak convulsions), 4 (prostration and strong convulsions). Intermediate values (0.5, 1.5, 2.5, or 3.5) were also given.

2.4. *Measurements of 5HT (or platelet translocation) and H*

Using 5HT as a marker for platelets, we quantitatively measured translocation of platelets into the lung and liver in mice.[12,17,18] Because the responses we analysed in the present study occur very rapidly (within 10 min), blood was collected by decapitation. One or two drops of the blood flowing from the neck were directly collected into a pre-weighed tube containing 3 ml of 0.4 M $HClO_4$, 0.1% N-acetyl cysteine–HCl, and 2 mM EDTA-2Na. After the tube had been reweighed, cells were destroyed by sonication, and each tube was cooled in an ice-bath. Lungs and livers were rapidly removed and kept in a jar with dry ice until needed. The determination of the 5HT level in the blood was carried out soon after the blood was collected, and the 5HT levels in the liver and lung were determined within 3 days of collection. After 5HT had been separated by column chromatography, it was measured fluorometrically as described elsewhere.[19] A portion of the blood samples described above was used for the determination of H, as described previously.[20]

2.5. *Statistical analysis*

Experimental values are given as mean ± standard deviation (S.D.). The statistical significance of differences was evaluated using Dunnett's multiple-comparison test after an analysis of variance (ANOVA). P-values less than 0.05 were considered to indicate significance. The difference in shock scores between two experimental groups was analysed using a Ridit test.[21]

3. Results and discussion

3.1. *Effects of Pi-LPS on sensitisation*

In mice sensitised with alum + OVA, histamine is released from mast cells a few (1–2) minutes of an OVA-challenge, and anaphylactic shock is induced within several (3–5) minutes of the challenge.[12] Fig. 1 shows the results from an experiment in which mice were sensitised with OVA alone, with alum + OVA, or with LPS + OVA. The shock induced by an OVA-challenge (2 mg/kg) was significantly more severe in mice sensitised with alum (a standard agent for immunization) + OVA than in mice sensitised with OVA alone. The severity of the shock seen in mice sensitised with LPS + OVA was similar to that seen in mice sensitised with alum + OVA. However, there were no significant differences in the elevated levels of blood histamine among these groups, even though histamine is believed to be an important allergic mediator and/or shock-inducer in anaphylaxis. These results suggest (a) that Pi-LPS possesses potent adjuvant activity (comparable to that of alum) for the sensitisation of mice to OVA, and (b) that in addition to histamine, another factor may be involved in the induction of anaphylactic shock. It should also be noted that there was no significant difference in the adjuvant effects of Pi-LPS at 0.1 and 1 mg/kg, suggesting that Pi-LPS induces its maximal adjuvant effect at around 0.1 mg/kg (2.5 μg/mouse). Incidentally, Funayama et al.[11] reported that an intragingival injection of Pi-LPS into mice induces inflammatory reactions in extraoral tissues, and that its effect is similar to that induced by intraperitoneal injection of LPS. The present findings suggest that oral bacterial LPS may have the potential to sensitise (or immunize) the host to foreign antigens.

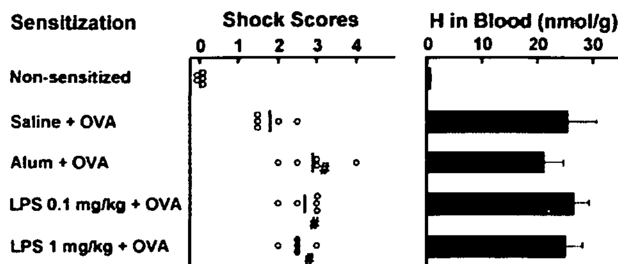

Fig. 1 – Adjuvant effect of Pi-LPS in the sensitisation of mice to the induction of anaphylactic shock and histamine-release from mast cells. Mice were sensitised (or immunized) with OVA alone, with alum + OVA, or with Pi-LPS (0.1 or 1.0 mg/kg) + OVA as described in Section 2. An OVA-challenge (2 mg/kg) was then delivered intravenously, and blood was taken by decapitation at 10 min after the OVA-challenge. The maximal shock score allocated to the shock signs seen within 10 min of the OVA-challenge was recorded for each mouse. The values shown for H are mean ± S.D. from 5 mice. #P < 0.05 vs. OVA alone (Ridit test).

3.2. *Effects of Pi-LPS on the development of anaphylactic shock*

Infection is thought to influence the extent of allergic inflammatory reactions[3]. We therefore examined the effect of a prior injection (i.p.) of Pi-LPS into sensitised mice (with alum + OVA) shortly before an OVA-challenge. In this experiment, the OVA-challenge was delivered at a dose of 1 mg/kg (less than in the experiment shown in Fig. 1) because a preliminary experiment showed that such LPS-treatment augmented the subsequent anaphylactic responses. Indeed, Pi-LPS, when injected (i.p.) 3 h before an OVA-challenge, significantly increased the severity of the shock (Fig. 2). In this

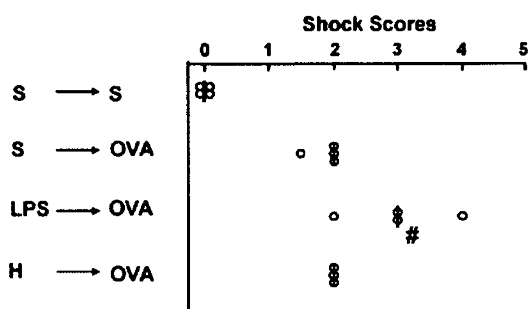

Fig. 2 – Augmenting effect of Pi-LPS on the anaphylactic shock induced by OVA-challenge. Mice were sensitised with alum + OVA as described in Section 2. Saline (S), LPS (0.1 mg/kg), or histamine (H, 10 μmol/kg) was given intraperitoneally to the mice, and 3 h later saline (S) or an OVA-challenge (1 mg/kg) was given. The maximal shock score allocated to the shock signs seen within 10 min of the OVA-challenge was recorded for each mouse. Histamine alone did not induce detectable shock signs in either sensitised or non-sensitised mice (data not shown). #P < 0.05 vs. S → OVA (Ridit test).

experiment, we also examined the effect of intravenous injection of histamine itself at a dose of 10 μmol/kg (i.v.). In mice, the weight of the total blood is around 7% of the body weight,[22] about 1.75 g in our mice. Thus, this dose of histamine (250 nmol/mouse) may, at least transiently, result in a level of histamine in the blood of around 143 nmol/g, a level much higher than those observed in the anaphylactic state illustrated in Fig. 1. However, even at this dose histamine itself induced no detectable shock signs within a 30-min observation period in mice sensitised with alum + OVA or in non-sensitised mice (data not shown). When sensitised mice were challenged with OVA at 3 h after the histamine injection, the severity of the shock was similar to that seen in control mice (Fig. 2). These results suggest that (i) histamine was not involved in the augmenting effect of a prior injection of LPS, even though in previous reports LPS had induced a significant amount of the histamine-forming enzyme, histidine decarboxylase, in various tissues at 3 h after its injection,[23,24] and (ii) histamine alone is not sufficient to induce anaphylactic shock in OVA-sensitised mice.

3.3. *Effects of prior treatment with Pi-LPS on the histamine-release and pulmonary platelet accumulation induced by antigen-challenge*

Using 5HT as a marker for platelets, we have shown that OVA-challenge induces in mice a rapid accumulation of platelets in the lung and liver (a particularly large amount in the lung), and that their degradation is involved in the subsequent anaphylactic shock.[12] We therefore examined the effects, in mice sensitised with alum + OVA, of giving LPS (0.1 mg/kg, i.p.) shortly (3 h) before OVA-challenge on the levels of 5HT in the lung and liver and on the level of histamine in the blood. In this experiment, we measured the levels of both 5HT and histamine at 1 min after a challenge with as little as 0.5 mg/kg OVA (12.5 μg/mouse), because platelets respond well to such a low dose (the platelet response occurring more rapidly than the release of histamine).[12] As shown in Fig. 3, the OVA-challenge increased 5HT in both lung and liver. LPS-treatment itself (LPS → S) had no detectable effect on the 5HT level in lung or liver, yet the 5HT increase in the lung was significantly higher in the LPS → OVA group than in the S → OVA group. However, there was no significant difference in the 5HT increase in the liver between these two groups. At 1 min after the OVA-challenge, histamine, too, was increased in the blood (note that this increase was much smaller than those shown for 10 min after OVA-challenge in Fig. 1), but there was no significant difference between the S → OVA and LPS → OVA groups. These results suggest that LPS-pretreatment may have a sensitisation effect on platelets, causing them to accumulate in the lung in greater numbers and leading to augmented anaphylactic shock. These results also support the interpretation mentioned above (viz. histamine is not by itself sufficient to induce anaphylactic shock in OVA-sensitised mice).

At present, we have no data indicating whether LPS sensitises platelets directly or indirectly. Although we have not examined the in vitro effects of Pi-LPS on platelets, this LPS, when injected into mice intravenously (but not intraperitoneally), has the ability to induce a rapid (within 5 min) accumulation of platelets in the lung and liver,[25] effects that

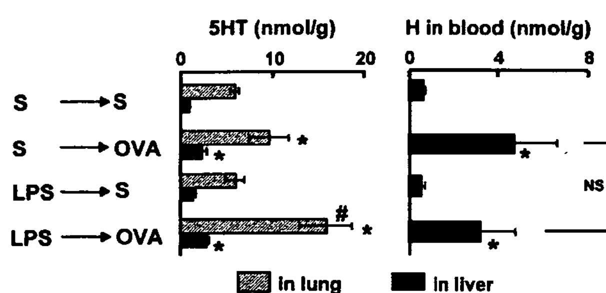

Fig. 3 – Effects of prior administration of Pi-LPS on the OVA-challenge-induced accumulation of platelets (i.e., 5HT) in the lung and liver and on histamine (H) release from mast cells. Mice were sensitised with alum + OVA as described in Section 2. Saline (S) or LPS (0.1 mg/kg) was given intraperitoneally to the mice, and 3 h later S or OVA (0.5 mg/kg) was given. Each mouse was decapitated at 1 min after the OVA-challenge, and blood, lung, and liver were taken as described in Section 2. The values for 5HT and H are mean ± S.D. from 5 mice. *$P < 0.05$ vs. S → S; #$P < 0.05$ vs. S → OVA; NS, not significant (Dunnett's multiple-comparison test).

are independent of TLR4[26] but dependent on the structure of the O-antigen region of LPS.[18]

3.4. *General discussion*

As described in Section 1, infections may prevent or reduce the development of allergic disorders such as atopy and asthma, a phenomenon sometimes referred to as the "hygiene hypothesis". This hypothesis suggests that a reduced frequency of infections, less severe infections, and/or prevention of infection (by the presence of excessively clean surroundings or by frequent use of antibiotics) can interfere with the maturation of Th1 immunity, leading to an enhancement of Th2 immune responses.[2,3,27] This idea is supported both by the finding that children who grow up on farms have fewer allergic disorders[3] and by the report that bacterial components (such as LPS and DNA) may be involved in promoting Th1 immunity.[1,3] However, several studies have shown that on the contrary, LPS may actually worsen allergic disorders.[28–33] Furthermore, in murine models, *E. coli* LPS has been shown to augment the IgE and IgG responses to allergens (such as latex allergen or cat allergen, administered intranasally or subcutaneously).[34,35] The present finding support the idea that exposure to oral bacterial LPS may be an important factor in sensitising the host to antigens and exacerbating allergic disorders (what we might call "the aggravation effect" of oral bacterial LPS). In "real-life", the two effects ("hygiene" and "aggravation" effects) could be present together, and it is impossible to say which might predominate at a given time.

We found that *E. coli* LPS has essentially the same property of promoting sensitization to OVA (data not shown). It is believed that the effects of LPS are exerted via TLR4.[36] However, many reports have suggested that LPS prepared from *Bacteroides*-related bacteria such as *Porphyromonas gingivalis* (*Pg*) and Pi by the hot-phenol water extraction method (Westphal's method) possess TLR2 agonistic activities. Recently, Ogawa et al. demonstrated that the activity of such *Pg*-LPS preparations is largely due to contamination of the LPS preparations with lipoprotein, the effects of which are mediated via TLR2.[37] The Pi-LPS used in our study, however, was prepared by the phenol-chloroform-petroleum ether extraction method (Galanos's method) and is almost devoid of activities (mitogenic and cytokine production activities *in vitro*) in cells isolated from TLR4-mutated C3H/HeJ mice.[15] Hence, the activity of the Pi-LPS reported here may be attributable to TLR4-mediated activity, although the activity of Pi-LPS is considerably weaker than that of *E. coli* LPS. However, we also have unpublished data suggesting a TLR4-independent *in vivo* activity of the present preparation of Pi-LPS. At present, we have no data to show whether or not TLR2 is also involved in the activity of our Pi-LPS preparation. To clarify this point, further studies will be required. In the present study, therefore, we did not refer to *E. coli* LPS.

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RESEARCH REPORTS

Biological

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Histamine Amplifies Immune Response of Gingival Fibroblasts

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ABSTRACT

Histamine is an important mediator in immune responses, but it is unclear whether periodontal tissues express histamine receptors and are able to respond to histamine. We hypothesized that histamine, inflammatory cytokines, and bacterial components released in inflamed periodontal tissues may be synergistically involved in periodontitis. The present study showed that human gingival fibroblasts mainly express histamine receptor H1R, and responded to histamine to produce interleukin (IL)-8. Stimulation of gingival fibroblasts with tumor necrosis factor-α, IL-1α, and lipopolysaccharide markedly induced IL-8 production, and the IL-8 production was synergistically augmented in the presence of or pre-treatment with histamine. Selective inhibitors of mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and phospholipase C (PLC) significantly inhibited the synergistic effect. These results indicate that histamine induces IL-8 production from gingival fibroblasts through H1R, and synergistically augments the inflammatory stimuli by amplification of the MAPK and NF-κB through H1R-linked PLC.

Abbreviations used: HDC, histidine decarboxylase; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; HR, histamine receptor; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; NF, nuclear factor; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; R, receptor; TLR, Toll-like receptor; α-MEM, alpha-minimum essential medium; FCS, fetal calf serum; RT-PCR, reverse-transcriptase polymerase chain-reaction; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; LDH, lactate dehydrogenase.

KEY WORDS: histamine, fibroblasts, inflammation, MAPK, NF-κB.

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INTRODUCTION

Histamine is an important mediator not only in allergic reactions but also in a variety of immune responses, including the production of inflammatory cytokines and the modulation of T-helper cell balance (Dy and Schneider, 2004). Histamine is released from stimulated mast cells or basophils. In addition, histamine is newly synthesized by a histamine-forming enzyme, histidine decarboxylase (HDC), in non-mast cells, and is released without being stored (Kahlson and Rosengren, 1968; Schayer, 1974; Endo, 1982). HDC is induced in various organs or tissues in response to a variety of inflammatory stimuli, including bacterial products, such as lipopolysaccharide (LPS), and inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-18, and IL-12 (Schayer, 1974; Endo, 1989; Yamaguchi *et al.*, 2000), indicating that histamine might also be induced in the inflamed gingiva of persons with periodontitis.

Histamine receptors (H1R, H2R, H3R, and H4R) belong to the G-protein-coupled receptor superfamily (Dy and Schneider, 2004). H1R and H2R are expressed in various cell types, while the expression of H3R and H4R is restricted to the brain and the hematopoietic cells, respectively. H1R is linked to the activation of phospholipase C (PLC) through the $G\alpha_{q/11}$ protein (Dy and Schneider, 2004). An H1R agonist, 2-[3-(fluoromethyl)phenyl]histamine, induces the activation of nuclear factor (NF)-κB, a ubiquitous transcription factor that is considered to play an important role in inflammatory processes, through $G\alpha_{q/11}$ and Gβγ (Bakker *et al.*, 2001). $G\alpha_{q/11}$ also stimulates mitogen-activated protein kinases (MAPKs), important mediators of signal transduction, through the protein kinase C- and Src family kinase-dependent signaling pathway (Nagao *et al.*, 1998). MAPKs are divided into at least 3 subfamilies: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK (Davis, 1994). TNF-α, IL-1, and LPS also activate NF-κB and MAPK pathways through respective receptors (Barton and Medzhitov, 2003; Wajant *et al.*, 2003; Subramaniam *et al.*, 2004).

Human gingival fibroblasts, the major constituent of gingival connective tissue, are heterogeneous (Lekic *et al.*, 1997; Sugawara *et al.*, 1998) and express immunological receptors (Rs), such as IL-1R (Kanda-Nakamura *et al.*, 1996), IL-2R (Ozawa *et al.*, 2004), TNFR (Butler *et al.*, 1994), CD14 (Watanabe *et al.*, 1996; Sugawara *et al.*, 1998), and Toll-like receptors (Tamai *et al.*, 2002). Stimulation of gingival fibroblasts with these cytokines and LPS induces the production of various cytokines, such as IL-1, IL-6, and IL-8 (Takada *et al.*, 1991; Watanabe *et al.*, 1996; Sakuta *et al.*, 1998; Sugawara *et al.*, 1998; Tamai *et al.*, 2002; Ozawa *et al.*, 2004). These observations indicate that gingival fibroblasts actively participate in immune responses and inflammatory processes.

It is unclear whether gingival fibroblasts express histamine receptors and are able to respond to histamine, but it is conceivable that, in inflamed periodontal tissues, histamine, inflammatory cytokines, and bacterial components are released together, and may be orchestrating immune

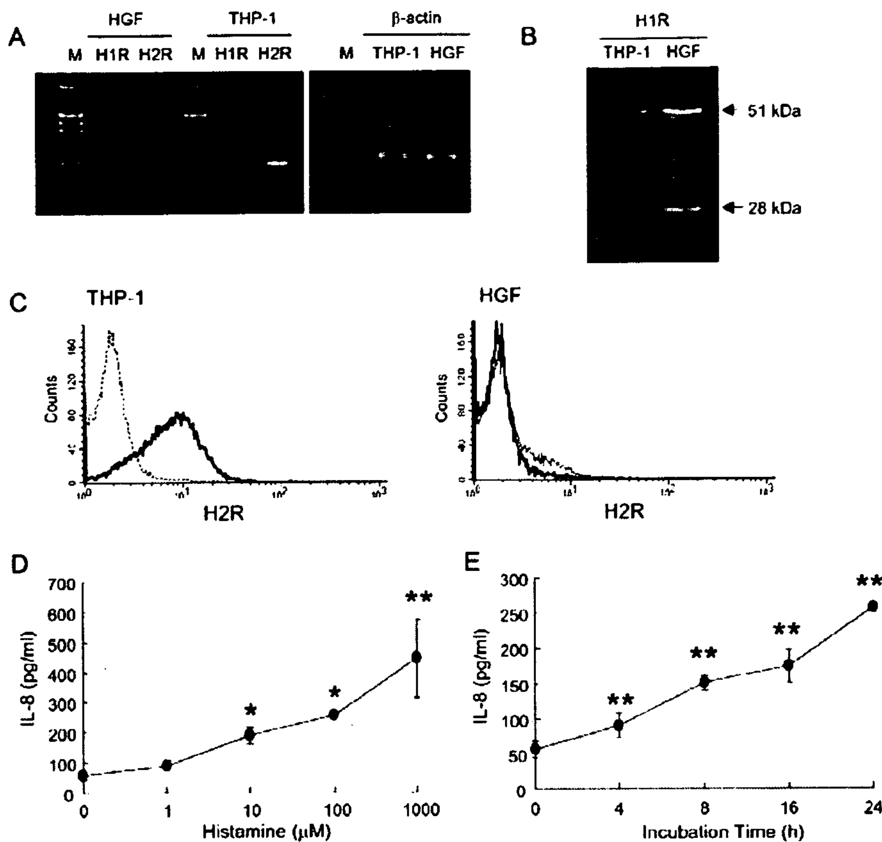

Figure 1. Expression of H1R in human gingival fibroblasts (HGF) and IL-8 secretion from the cells in response to histamine. (A) Total RNA was extracted from confluent gingival fibroblasts. THP-1 cells were used as a positive control. cDNA was prepared and analyzed for the mRNA expression of β-actin, H1R, and H2R by RT-PCR. M, molecular-weight marker. (B) Cell membrane fraction was separated from gingival fibroblasts and THP-1 cells and mixed with Laemmli sample buffer. Samples (equivalent to 10^6 cells each) were then subjected to Western blotting with rabbit anti-human H1R antibody. (C) Gingival fibroblasts and THP-1 cells were stained with 2 different rabbit anti-human H2R antibodies and analyzed by flow cytometry. Results in A, B, and C are representative of those from six donors with similar results. (D,E) Gingival fibroblasts were stimulated with the indicated concentrations of histamine for 24 hrs (D) or with 100 μmol/L of histamine for the time indicated (E). Supernatants were then collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. $^*p < 0.05$, and $^{**}p < 0.01$ compared with the unstimulated control (medium alone).

responses and inflammatory processes. To examine this hypothesis, we used TNF-α, IL-1α, and LPS as major inflammatory stimuli and measured the secretion of IL-8, one of the major inflammatory mediators, from gingival fibroblasts, in response to the inflammatory stimuli with or without histamine. We also used specific signaling inhibitors to elucidate the signaling pathway.

MATERIALS & METHODS

Reagents

Human recombinant IL-1α and TNF-α were supplied by Dainippon Pharmaceutical (Osaka, Japan). Dimaprit was obtained from Wako (Osaka, Japan). Signaling inhibitors were obtained from Calbiochem (San Diego, CA, USA). LPS from *Escherichia coli* O55:B5, 2-pyridylethylamine, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Cells and Cell Culture

Human gingival fibroblasts were prepared from explants of normal gingival tissues of persons with adult periodontitis undergoing periodontal surgery after providing informed consent, as previously described (Sugawara *et al.*, 1998). Cells were used at passages 5 through 8. The Ethical Review Board of Tohoku University Graduate School of Dentistry (Sendai, Japan) approved the experimental procedures. Human monocytic THP-1 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and used as positive controls.

Reverse-transcriptase/Polymerase Chain-reaction (RT-PCR)

Total RNA was isolated from cells (2 x 10^6 cells) by means of a Total RNA Isolation kit (Isogen, Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. RT-PCR for H1R, H2R, and β-actin was performed as previously described (Gutzmer *et al.*, 2002; Butch *et al.*, 1993). Amplified samples were visualized after electrophoresis on 2% agarose gels and staining with ethidium bromide, and photographed under ultraviolet light.

Western Blotting and Flow Cytometry

Western blotting of cell membrane fractions and flow cytometric analysis with FACSCalibur and CELLQuest software (BD Biosciences, San Diego, CA, USA) were performed as described previously (Sugawara *et al.*, 1998), with the following antibodies: rabbit anti-human H1R (Chemicon International, Temecula, CA, USA), rabbit anti-human H2R (Alphadiagnostic International, San Antonio, TX, USA; GeneTex, San Antonio, TX, USA).

Detection of IL-8

Gingival fibroblasts (10^5 cells/500 μL) were seeded in alpha-minimum essential medium (α-MEM) with 10% fetal calf serum (FCS) in 24-well plates (BD Labware, Lincoln Park, NJ, USA). After incubation for one day, cells were washed with α-MEM 3 times, and test-stimulants were added in 500 μL of α-MEM with 1% FCS for the time indicated. For the inhibition experiments, gingival fibroblasts in 24-well plates were pre-incubated with inhibitors for 30 min to 1 hr at 37°C, and were then stimulated with test-stimulants at 37°C. After the incubation, the levels of IL-8 in the supernatants were determined with an OptEIA human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

We replicated all of the experiments in this study at least 3 times to confirm the reproducibility of the results. Experimental values are given as means ± standard deviation (SD) of triplicate assays. The statistical significance of differences between the 2 means was evaluated by a one-way analysis of variance by the Bonferroni or Dunnett method, and values of $p < 0.05$ were considered significant.

RESULTS

Expression of H1R in Gingival Fibroblasts and Secretion of IL-8 in Response to Histamine

We first examined whether human gingival fibroblasts express histamine receptors and are able to respond to histamine. Gingival fibroblasts constitutively expressed H1R and H2R mRNAs, as assessed by RT-PCR (Fig. 1A), and H1R protein, as assessed by Western blotting (Fig. 1B). However, expression of H2R protein was not clearly detected in gingival fibroblasts by Western blotting and flow cytometry (Fig. 1C and data not shown). THP-1 cells were used as a positive control. Stimulation of gingival fibroblasts with histamine at 10 μmol/L significantly induced IL-8 production, and the production was further increased at higher concentrations (Fig. 1D). A time kinetic study showed that the histamine-stimulated IL-8 production from gingival fibroblasts was time-dependent, and the production was highest at 24 hrs (Fig. 1E).

Histamine Synergistically Augments IL-8 Production from Gingival Fibroblasts Induced by TNF-α, IL-1, and LPS

Stimulation of gingival fibroblasts with an inflammatory cytokine TNF-α at 1 and 10 ng/mL for 6 hrs markedly induced IL-8 production from gingival fibroblasts, and the IL-8 levels were high compared with those induced by histamine alone at 100 μmol/L (Med→His *vs.* Med→TNF) (Fig. 2A). Furthermore, the TNF-induced IL-8 production was synergistically augmented in the presence of 100 μmol/L histamine (Med→His *vs.* Med→His+TNF), and the IL-8 levels induced by 1 ng/mL of TNF-α and 100 μmol/L histamine were comparable with those induced by 10 ng/mL of TNF-α alone. Pre-treatment of gingival fibroblasts with histamine for 2 hrs also amplified the IL-8 production by subsequent stimulation with TNF-α (His→TNF).

The synergism was observed with another inflammatory cytokine IL-1α and histamine (Fig. 2B). Stimulation of gingival fibroblasts with LPS for 6 hrs induced low levels of IL-8 (about 50-100 ng/mL), and histamine showed only an additive effect on LPS-induced IL-8 production (data not shown). However, 22-hour stimulation with LPS in the presence of histamine or following pre-treatment with histamine synergistically induced IL-8 production from gingival fibroblasts (Fig. 2C).

Involvement of Functional H1R in the Synergistic Effect

We next examined whether H1R is functionally involved in the histamine response and the synergistic effect. To examine this, we used H1R agonist 2-pyridylethylamine and H2R agonist dimaprit instead of histamine. The IL-8 production from gingival fibroblasts was significantly augmented in the presence of the H1R agonist at 0.1 and 1 mmol/L (Fig. 3A). In contrast, the H2R agonist at 0.1 mmol/L showed no effect on IL-8 production, and the IL-8 levels were further decreased at 1 mmol/L, due to the toxicity at this concentration, as assessed by lactate dehydrogenase activity. TNF-induced IL-8 production was also markedly augmented by the H1R agonist, which was higher than that of histamine at 100 μmol/L; whereas, the H2R agonist showed no effect (Fig. 3B). Similar results were obtained with IL-1α and LPS as stimulants (data not shown). These results indicate that histamine stimulates gingival fibroblasts through H1R, and that H1R is involved in the inflammatory stimuli-induced synergistic effect.

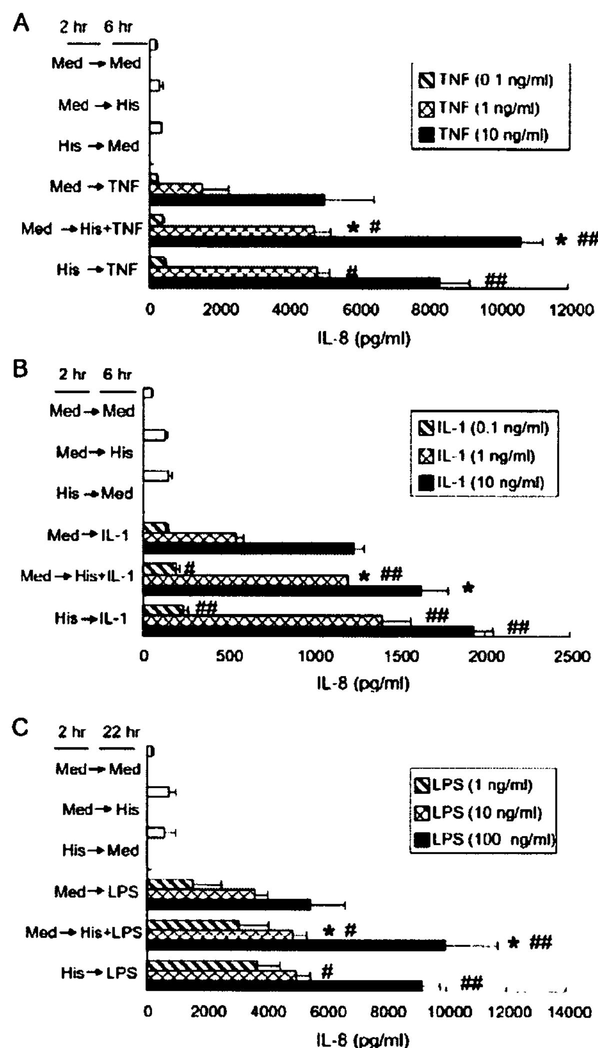

Figure 2. Synergistic effect of histamine for IL-8 production from gingival fibroblasts stimulated with TNF-α, IL-1α, and LPS. Gingival fibroblasts were incubated with 100 μmol/L of histamine (His) or medium (Med) for 2 hrs. The cells were then stimulated with TNF-α **(A)**, IL-1α **(B)**, or LPS **(C)** at the indicated concentrations in the presence or absence of histamine at 100 μmol/L for 6 hrs for TNF-α and IL-1α or 22 hrs for LPS. After the incubation, supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. *$p < 0.01$ compared with Med→His. #$p < 0.05$ and ##$p < 0.01$ compared with Med→TNF-α, Med→IL-1α, or Med→LPS.

Involvement of MAPK, NF-κB, and PLC in the Synergistic Effect

Finally, the possible involvement of MAPK, NF-κB, and PLC

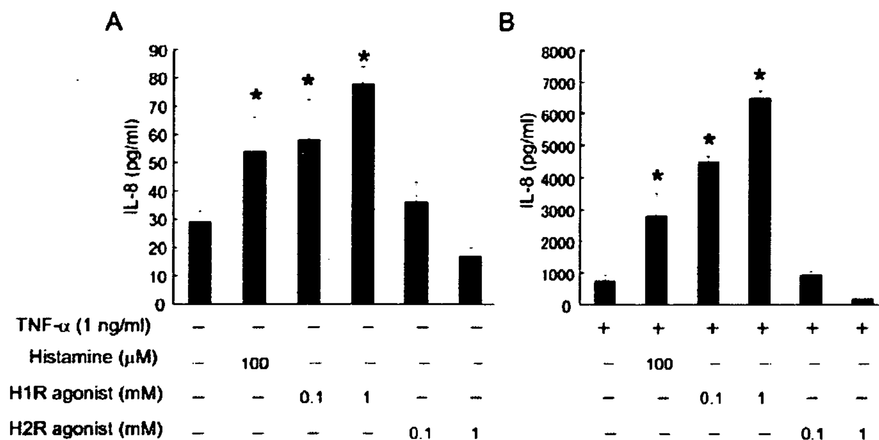

Figure 3. Involvement of functional H1R in the synergistic effects. Gingival fibroblasts were incubated in medium (A) or with TNF-α (1 ng/mL) (B) in the presence or absence of histamine, the H1R agonist 2-pyridylethylamine, or the H2R agonist dimaprit at the indicated concentrations for 8 hrs. After incubation, the supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. *$p < 0.01$ compared with medium or TNF-α alone.

in the synergistic effect was examined. Histamine-induced IL-8 production was significantly inhibited by inhibitors of MEK (PD98059), JNK (SP600125), p38 MAPK (SB203580), and NF-κB (pyrrolidine dithiocarbamate, PDTC) (Fig. 4A). The TNF-induced IL-8 production was also significantly suppressed by each of the inhibitors, and was markedly inhibited by a combination of these inhibitors (Fig. 4B). No lactate dehydrogenase activity was detected in all of the supernatants for ELISA (data not shown), indicating that the cell membrane was not damaged by the treatment with inhibitors. The synergistic IL-8 production induced by histamine and TNF-α was also significantly inhibited by these inhibitors (Fig. 4B), indicating that the synergistic effect was mediated by the activation of MAPKs and NF-κB.

H1R is linked to PLC activation through $G\alpha_{q/11}$ (Dy and Schneider, 2004). To examine whether PLC is involved in the synergistic effect, we stimulated gingival fibroblasts with histamine and TNF-α in the presence of PLC inhibitor U73122 or the control compound U73343. The inhibition of PLC suppressed IL-8 production induced by histamine and TNF-α to the level of TNF-α alone, whereas the control compound did not suppress IL-8 production (Fig. 4C). Similar results were obtained with IL-1α and LPS as stimulants (data not shown). These results indicate that $G\alpha_{q/11}$-coupled PLC is involved in the synergism, and that TNF-induced IL-8 production is not mediated by the activation of PLC.

DISCUSSION

Mast cells are detected in healthy gingiva and gingivitis lesions (Gemmell *et al.*, 2004) and have been found in high numbers in chronically inflamed gingival tissue (Steinsvoll *et al.*, 2004), indicating that the source of histamine is gingival mast cells. In addition, histamine is synthesized by histidine decarboxylase and released without being stored from other cell types, such as monocytes/macrophages, neutrophils, and vascular endothelial cells (Kahlson and Rosengren, 1968; Schayer, 1974; Endo, 1982). Therefore, gingival histamine may also be derived from a non-mast-cell origin. The increase in salivary histamine levels is correlated with severity of periodontitis (Venza *et al.*, 2006). These observations suggest that histamine might also be released in the inflamed gingiva of persons with periodontitis. Periodontitis is caused by Gram-negative periodontopathic bacteria, and inflamed gingival epithelial cells, as well as infiltrated lymphocytes, appear to express several inflammatory cytokines, IL-1, IL-6, IL-8, and TNF-α (Lundqvist *et al.*, 1994). Therefore, the histamine synergism shown in this study likely occurs *in vivo*, and histamine in periodontal tissues potentially modulates the initiation and development of periodontitis.

The present study showed that histamine amplifies inflammatory stimuli from TNF-α, IL-1, and LPS, in gingival fibroblasts. The IL-8 levels induced by these inflammatory stimuli with histamine were comparable with those induced by a ten-fold concentration of the inflammatory stimuli alone, indicating that histamine augments the activity of the inflammatory stimuli at the site of inflammation approximately ten-fold as compared with that of the stimuli alone.

Gingival fibroblasts mainly expressed H1R. Gingival fibroblasts also expressed H2R mRNA, but the expression of H2R protein was not clearly detected in the cells (data not shown), indicating that translational and post-transcriptional mechanisms modify or degrade the H2R mRNA transcript. H1R are linked $G\alpha_{q/11}$ proteins (Dy and Schneider, 2004), and $G\alpha_{q/11}$ stimulate NF-κB (Bakker *et al.*, 2001) and MAPKs (Nagao *et al.*, 1998). Signaling through TNFR, IL-1R, and the LPS receptor also activates both cascades (Barton and Medzhitov, 2003; Wajant *et al.*, 2003; Subramaniam *et al.*, 2004), and this study confirmed this evidence. Furthermore, histamine activates MAPK and NF-κB signaling cascades *via* H1R by specific inhibitors and histamine receptor agonists. Synergistic IL-8 production was also significantly suppressed by the inhibition of NF-κB and MAPKs. It has been reported that peptidoglycan induces the activation of NF-κB *via* MAPKs in murine macrophages (Chen *et al.*, 2004). However, the inhibition of MAPKs in stimulated gingival fibroblasts did not result in the inhibition of NF-κB (data not shown). These observations indicate that the amplification of MAPKs and that of NF-κB are equally involved in synergism in gingival fibroblasts.

The principal mechanism of H1R activation is through $G\alpha_{q/11}$, resulting in the activation of PLC (Dy and Schneider, 2004). The inhibition of PLC suppressed the production of IL-8 induced by histamine and TNF-α to the levels of TNF-α alone. The results indicate that histamine activates PLC through H1R, and consequently amplifies the MAPK and NF-κB pathway signaling induced by the inflammatory stimuli.

This study showed that the secretion of IL-8, a major product from gingival fibroblasts, is mediated by the activation of MAPKs and NF-κB, which was consistent with the report from a previous study (Hoffmann *et al.*, 2002). IL-8 mainly

activates neutrophils, promoting their recruitment. IL-8 is also a chemoattractant for other cell types, such as basophils, T-cells, and natural killer cells, and enhances the permeability of endothelial cells (Baggiolini *et al.*, 1997). It has been reported that histamine causes limited inhibition of neutrophil chemotaxis (direct motility) while stimulating chemokinesis (random motility) (Seligmann *et al.*, 1983). Therefore, IL-8 from gingival fibroblasts and histamine in periodontal tissues may control neutrophil locomotion and play an important role in the control of inflammation at the periodontitis site. Since TNF-α, IL-1, and LPS stimulate histamine production through mast-cell-independent HDC induction (Wu *et al.*, 2004), it is also conceivable that histamine is induced by inflammatory cytokines and LPS, and, in turn, augments the biological functions of inflammatory cytokines and LPS.

In conclusion, the present study showed that gingival fibroblasts secrete IL-8 in response to histamine through H1R, and that histamine synergistically augments IL-8 secretion induced by TNF-α, IL-1α, and LPS by the amplification of the MAPK and NF-κB pathway through H1R-linked PLC. Recent studies also reported that an H2R antagonist, cimetidine, prevents periodontitis in a rat model (Hasturk *et al.*, 2006), and that topical cimetidine oral rinse enhances the antibacterial function of human crevicular neutrophils (Van Dyke *et al.*, 2005), although the effect of H1R antagonists on periodontitis was not reported. Therefore, control of histamine receptors at inflammatory sites might be beneficial in the regulation of periodontitis.

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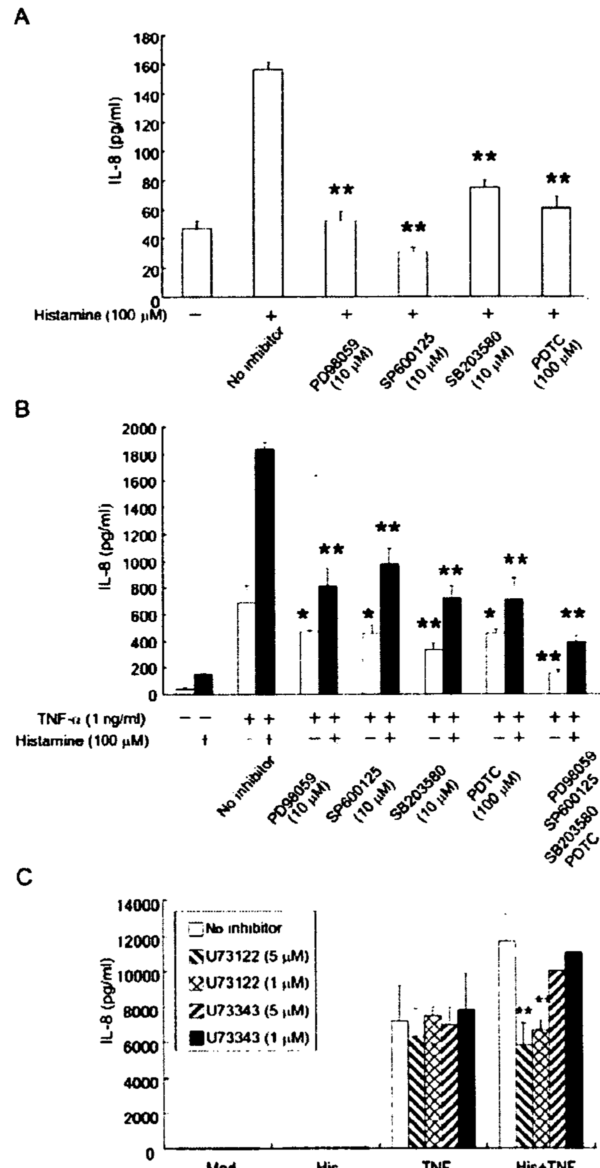

Figure 4. Involvement of MAPK, NF-κB, and PLC on the synergistic effects. **(A,B)** Gingival fibroblasts were pre-treated with or without PD98059, SP600125, SB203580, and/or PDTC at the dose indicated for 1 hr. The cells were then incubated with or without histamine (100 μmol/L) alone (A) or in the presence or absence of histamine (100 μmol/L) and TNF-α (1 ng/mL) (B) for 8 hrs. **(C)** Gingival fibroblasts were pre-treated with or without U73122 or U73343 at the dose indicated for 30 min. The cells were then incubated in the presence or absence of histamine (100 μmol/L) and TNF-α (10 ng/mL) for 8 hrs. After incubation, the supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. *$p < 0.05$, and **$p < 0.01$ compared with no inhibitor.

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Biotin deficiency up-regulates TNF-α production in murine macrophages

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Abstract: Biotin, a water-soluble vitamin of the B complex, functions as a cofactor of carboxylases that catalyze an indispensable cellular metabolism. Although significant decreases in serum biotin levels have been reported in patients with chronic inflammatory diseases, the biological roles of biotin in inflammatory responses are unclear. In this study, we investigated the effects of biotin deficiency on TNF-α production. Mice were fed a basal diet or a biotin-deficient diet for 8 weeks. Serum biotin levels were significantly lower in biotin-deficient mice than biotin-sufficient mice. After i.v. administration of LPS, serum TNF-α levels were significantly higher in biotin-deficient mice than biotin-sufficient mice. A murine macrophage-like cell line, J774.1, was cultured in a biotin-sufficient or -deficient medium for 4 weeks. Cell proliferation and biotinylation of intracellular proteins were decreased significantly in biotin-deficient cells compared with biotin-sufficient cells. Significantly higher production and mRNA expression of TNF-α were detected in biotin-deficient J774.1 cells than biotin-sufficient cells in response to LPS and even without LPS stimulation. Intracellular TNF-α expression was inhibited by actinomycin D, indicating that biotin deficiency up-regulates TNF-α production at the transcriptional level. However, the expression levels of TNF receptors, CD14, and TLR4/myeloid differentiation protein 2 complex were similar between biotin-sufficient and -deficient cells. No differences were detected in the activities of the NF-κB family or AP-1. The TNF-α induction by biotin deficiency was down-regulated by biotin supplementation in vitro and in vivo. These results indicate that biotin deficiency may up-regulate TNF-α production or that biotin excess down-regulates TNF-α production, suggesting that biotin status may influence inflammatory diseases. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: *cytokine · inflammation · nutrition*

INTRODUCTION

Biotin is a water-soluble vitamin of the B complex found in all organisms [1]. Biotin functions as a cofactor of five carboxylases—pyruvate carboxylase (EC 6.4.1.1), two forms of acetyl-CoA carboxylase (ACC1 and ACC2; EC 6.4.1.2), propionyl-CoA carboxylase (PCC; EC 6.4.1.3), and methylcrotonoyl-CoA carboxylase (EC 6.4.1.4)—enzymes that catalyze the metabolism of glucose, amino acids, and fatty acids. As these biotin-dependent enzymes are indispensable for cellular metabolism, biotin starvation or deficiency is potentially lethal [2]. Biotin is covalently attached to specific lysine residues of carboxylases by the enzyme holocarboxylase synthetase (HCS; EC 6.3.4.10) [2]. During the turnover of carboxylases, biotin is released from biotinylated peptides by biotinidase (EC 3.5.1.12) and recycled in the biotinylation of new carboxylases [2]. The defect of this biotin cycle causes a neonatal form of life-threatening ketoacidosis and organic acidemia, known as multiple carboxylase deficiency [2].

In addition to this classical function as a cofactor of carboxylases, biotin is involved in various cellular events. It was reported that biotin regulates the mRNA expression of HCS and biotin-dependent carboxylases via a cGMP-dependent pathway [3]. Biotin regulates transcription factors, such as NF-κB, specificity protein 1 (Sp1), and Sp3, in human T cell line Jurkat cells [4, 5]. Moreover, biotinylation of histones in human cells was also reported [6]. In a human hepatoblastoma cell line, biotin regulates the expressions of asialoglycoprotein receptor and insulin receptor at the post-transcriptional level [7]. These reports clearly indicated that biotin regulates the various cellular events at transcriptional and post-transcriptional levels.

Immunological effects of biotin have also been studied. In vitro biotin supplementation induces IL-2Rγ expression and decreases the net secretion of IL-2 from Jurkat cells [8, 9]. In vivo supplementation of a pharmacologic dose of biotin decreases the proliferation rate of PBMC and the release of IL-1β and IL-2 [10]. Moreover, biotin deficiency changes the number and subpopulations of spleen lymphocytes and blocks thymocyte maturation in mice [11, 12]. On the other hand, contradictory results have been reported, namely that lymphocyte subpopulations, mitogen-induced cytokine production, IgG responses, and NK cell activity do not differ significantly be-

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tween mild to moderately biotin-deficient and biotin-sufficient rats [13]. Therefore, the immunological effects of biotin have not been clarified.

Moderately severe biotin deficiency causes alopecia and scaly erythematous dermatitis [11, 14, 15]. In addition to applications as a dietary supplement, biotin is prescribed for chronic dermatitis. It was reported that biotin has a therapeutic effect on pustulosis palmaris et plantaris, a type of chronic dermatitis that is restricted to the palms and soles and is related to metal allergy [16, 17]. Moreover, Makino et al. [18] reported that serum biotin levels are significantly lower in atopic dermatitis patients than in healthy subjects. These reports suggest that biotin deficiency is involved in inflammatory diseases. However, few reports are available about the biological roles of biotin in inflammatory responses.

In this study, we investigate the in vivo effects of biotin deficiency using a mouse model of LPS-induced TNF-α production. We also investigated the in vitro effects of biotin deficiency on the production of TNF-α by the murine macrophage cell line J774.1. We showed that biotin status affects the production of TNF-α, and biotin-supplementation down-regulated it in vivo and in vitro.

MATERIALS AND METHODS

Reagents

LPS from *Escherichia coli* O55:B5, prepared by Westphal's method, was purchased from Difco Laboratories (Detroit, MI, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Mice

Female BALB/c mice (4 weeks old), obtained from the Institute for Experimental Animals of the Tohoku University Graduate School of Medicine (Sendai, Japan), were used for the experiments. Mice were divided into two groups: biotin-sufficient and -deficient groups, which received a basal diet (AIN-76, containing 0.8 mg *d*-biotin per kg) or a biotin-deficient (biotin-free) AIN-76 diet (Nosan Corp., Yokohama, Japan), respectively. Preliminary experiments showed that 8-week feeding led to a significant decrease in the serum biotin level in mice. For in vivo biotin supplementation, biotin-deficient mice were provided with biotin-supplemented drinking water (15 μM) for 2 weeks. The dose of biotin was based on the dose of human biotin therapy (10–40 mg/day) and drinking volume of mice (5 ml/day) [19, 20]. The Ethical Board for Nonhuman Species of the Tohoku University Graduate School of Medicine approved the experimental procedure followed in this study.

Measurement of biotin in serum

Biotin in serum was purified by HPLC and measured by ELISA, as originally described by Mock [21]. Briefly, serum (1 ml; the serum was pooled from five mice) was ultrafiltrated using a centrifugal ultrafiltration unit (a MW cutoff of 30 kDa) at 1500 *g* for 90 min at 4°C. The filtrates were adjusted to pH 2.5 by 6 M HCl and then separated by reversed-phase HPLC on a Capcelpak AG120A C18 (5 μm, 4.6×250 mm, Shiseido, Tokyo, Japan) column at a flow rate of 1.0 ml/min at 40°C. Elution was carried out with solution A [0.1% trifluoroacetic acid (TFA)] and solution B (80% acetonitrile in 0.8% TFA). The linear gradient was started at solution A 100% and solution B 0% and was reached at solution A 81% and solution B 19% at 30 min and then solution A 0% and solution B 100% at 60 min. The column eluent was collected every minute in test tubes and dried by a centrifugal concentrator. After drying, each fraction was dissolved in 500 μl H_2O. From preliminary experiments with commercial biotin standard, we confirmed that biotin elutes at 32 min. For ELISA, 96-well, flat-bottomed plates (Nunc-Immuno modules, MaxiSorp, Nalge-Nunc International, Rochester, NY, USA) were coated with biotinyl-BSA, synthesized with BSA and biotin N-hydroxysuccinimide ester (Pierce Biotechnology, Inc., Rockford, IL, USA), and then blocked with 0.01% (wt/vol) BSA. Samples (100 μL) and 50 μl HRP-conjugated avidin (Calbiochem, Darmstadt, Germany) were mixed and incubated for 2 h at room temperature. Then, 100 μl of the mixture was transferred to a well of a biotinyl-BSA-coated plate and incubated for 4 h at room temperature. The ELISA was developed with 3,3′,5,5′ tetramethylbenzidine (substrate reagent set, BD Biosciences, San Diego, CA, USA) as a substrate. The detection range of biotin was 3–10,000 nM.

Measurement of PCC activity

PCC activity was measured as originally described by Zempleni et al. [22]. Briefly, the liver was homogenized on ice in buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM 2-ME, and 0.25 M sucrose and centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was used as a crude enzyme fraction. For PCC assay, a 5-μl tenfold-diluted crude enzyme fraction was incubated with a 95-μl reaction mixture for 5 min at room temperature. The reaction mixture consisted of 100 mM Tris-HCl (pH 8.0), 0.75 mM DTT, 6 mM $MgCl_2$, 3.14 mM Na_2-ATP, 100 mM KCl, 1% Triton X-100, 1 mM propionyl-CoA, and 3.5 mM $NaH[^{14}C]O_3$ (2.11 kBq/nmol). The reaction was stopped by the addition of 30 μl 1 M $HClO_4$. After centrifugation at 1000 *g* for 15 min, 100 μl aliquot was transferred into a vial and dried. Finally, the sample was resolved in a scintillation cocktail, and the bound [^{14}C]bicarbonate was quantified by a liquid scintillation counter. PCC activity was expressed in units: 1 unit = 1 nmol HCO_3^- fixed/min/mg protein.

Measurement of TNF-α in serum

LPS (1 μg/kg) was injected i.v. into mice. After 90 min, blood was collected and coagulated on ice for 1 h, and sera were recovered by centrifugation. The amounts of TNF-α in sera were measured with an OptEIA mouse TNF-α (Mono/Mono) set (BD Biosciences).

Biotin depletion from FCS

Biotin in FCS was depleted with immobilized avidin-agarose (Pierce Biotechnology, Inc.). Briefly, FCS (Tissue Culture Biologicals, Tulare, CA, USA) was mixed with avidin-agarose and gently stirred overnight at 4°C. After centrifugation, FCS was sterilized by filtration (0.22 μm pore size) and stored at −30°C until use. Biotin depletion was confirmed by ELISA as described above. The biotin concentration in FCS was 8.6 ± 0.25 nM, and that in biotin-deficient FCS was not detectable (less than 3 nM).

Cells and cell culture

J774.1, a murine macrophage-like cell line, was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. J774.1 cells were grown in biotin-sufficient and -deficient medium. The biotin-sufficient medium was RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) containing *d*-biotin (0.2 μg/ml) supplemented with 10% FCS. The biotin-deficient medium was biotin-free RPMI 1640 supplemented with 10% biotin-deficient FCS.

Western blotting

J774.1 cells were lysed in SDS-PAGE sample buffer. Cell lysates were separated by SDS-PAGE under reducing conditions. After SDS-PAGE, gel proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was blocked for 1 h with 3% (wt/vol) skim milk and 0.05% Tween 20 in PBS and incubated with HRP-conjugated avidin (Calbiochem). After washing, the blot was analyzed with SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Inc.) and a Chemi Imager (Alpha Innotech Corp., San Leadro, CA, USA).

[^{3}H] TdR incorporation assay

J774.1 cells were suspended in RPMI 1640 with 10% FCS and seeded in a 96-well, flat-bottomed plate (Nalge-Nunc International) at the indicated cell

numbers. The cells were incubated at 37°C for 3 h, and each well was pulsed with 1 kBq [^{3}H] TdR (MP Biomedicals, Irvine, CA, USA) for 2 h. After the pulse, 100 μl 3% Triton X-100 was added to each well, and the cells were harvested onto a glass fiber filter. The counts of [^{3}H] TdR per minute (cpm) were measured with a liquid scintillation counter (Packard Instrument Company, Meriden, CT, USA).

Measurement of TNF-α in culture supernatants of J774.1 cells

J774.1 cells were suspended in RPMI 1640 with 10% FCS and seeded in a 96-well flat-bottomed plate at 2 × 10^5 cells/200 μl/well. The cells were stimulated with LPS for 24 h at 37°C. The amount of TNF-α in the culture supernatant was measured with an OptEIA mouse TNF-α (Mono/Mono) set (BD Biosciences).

Flow cytometry

For TNF-α staining, J774.1 cells were seeded in a six-well, flat-bottomed plate (Nalge-Nunc International) at 1 × 10^6 cells/2 ml/well with RPMI 1640 with 10% FCS. The cells were cultured for 6 h at 37°C in the presence or absence of 1 μg/ml actinomycin D (Act-D). Then, the cells were stained with FITC-conjugated anti-mouse TNF mAb (clone MP6-XT22, BD Biosciences) for cell surface cytokine staining. For intracellular cytokine staining, the cells were incubated with a protein transport inhibitor (GolgiPlug, BD Biosciences). After fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences), the cells were stained with FITC-conjugated anti-mouse TNF mAb. For cell surface receptor staining, J774.1 cells were stained with PE-conjugated anti-mouse CD120a (TNFR type I) mAb (clone 55R-286, BioLegend, San Diego, CA, USA), PE-conjugated anti-mouse CD120b (TNFR type II) mAb (clone TR75-89, AbD Serotec, Oxford, UK), PE-conjugated anti-mouse CD14 mAb (clone rmC5-3, BD Biosciences), or FITC-conjugated anti-TLR4/myeloid differentiation protein 2 (MD2) complex mAb (clone MTS510, Stressgen Bioreagents, Ann Arbor, MI, USA). Expression of each molecule was measured using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Measurement of lactate dehydrogenase (LDH) activity

For the quantification of plasma membrane damage, LDH activity in the culture supernatants of J774.1 cells was measured with a cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

Cells were lysed in 1 ml Isogen (Nippon Gene, Toyama, Japan), and total RNA was extracted as described in the instruction manual. Total RNA was dissolved in 30 μl diethyl pyrocarbonate-treated water (Nippon Gene) and incubated at 65°C for 10 min. cDNA synthesis was carried out with a first-strand cDNA synthesis kit (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). Real-time PCR was performed with a LightCycler FastStart DNA Master SYBR Green I and a LightCycler 1.5 system (Roche Diagnostics). The primers used for PCR were as follows: TNF-α, forward 5′-AGCCTCTTCTCATTCCTGC-3′ and reverse 5′-GGAGGCCATTTGGGAACT-3′; β-actin, forward 5′-CGTTGACATCCGTAAAGACCTC-3′ and reverse 5′-AGCCACCGATCCACACAGA-3′ (Nihon Gene Research Labs Inc., Sendai, Japan). The PCR conditions were 35 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The product sizes for TNF-α and β-actin were 106 bp and 173 bp, respectively. The mRNA expression levels were expressed as relative units after normalization by the β-actin level. The specificity of the PCR was confirmed by the molecular weight of the products and melting curve analysis for each data point. PCR products were electrophoresed using 3% agarose (NuSieve 3:1 agarose, BMA, Rockland, ME, USA). After staining with ethidium bromide, amplified DNA bands were analyzed with a Chemi Imager.

Measurement of NF-κB and AP-1 activity

Activities of NF-κB family and AP-1 in nuclear extracts were measured with a NF-κB assay kit specific for the p65, p50, and RelB, p52 subunits, and an AP-1 assay kit specific for the phospho c-Jun, according to the manufacturer's instructions (Active Motif, Carlsbad, CA), respectively. The nuclear extractions were performed with a nuclear extract kit (Active Motif), according to the manufacturer's instructions. Protein concentrations of each fraction were determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc.). Each activity was expressed as absorbance at 450 nm ($A_{450\ nm}$) per 100 μg protein.

Data analysis

All of the experiments in this study were performed at least three times to confirm the reproducibility of the results. The data shown are representative results. Experimental values are given as the mean ± SD of triplicate assays. Statistical analysis was performed with the unpaired *t*-test or one-way ANOVA using Dunnett's method, and $P < 0.05$ was considered significant.

RESULTS

Augmentation of LPS-induced serum TNF-α levels in biotin-deficient mice

As it is well known that LPS induces the elevation of the serum concentration of TNF-α in mice, we first examined the in vivo effects of biotin deficiency on LPS-induced TNF-α production. Mice were fed biotin-sufficient or -deficient diets. After 8 weeks of feeding, the serum concentrations of biotin were significantly ($P<0.001$) lower in biotin-deficient mice than biotin-sufficient mice (**Fig. 1A**). However, PCC activities in the liver were comparable between biotin-sufficient and -deficient mice (sufficiency: 2.99±0.49 nmol HCO_3^- fixed/min/mg protein; deficiency: 2.80±0.34 nmol HCO_3^- fixed/min/mg pro-

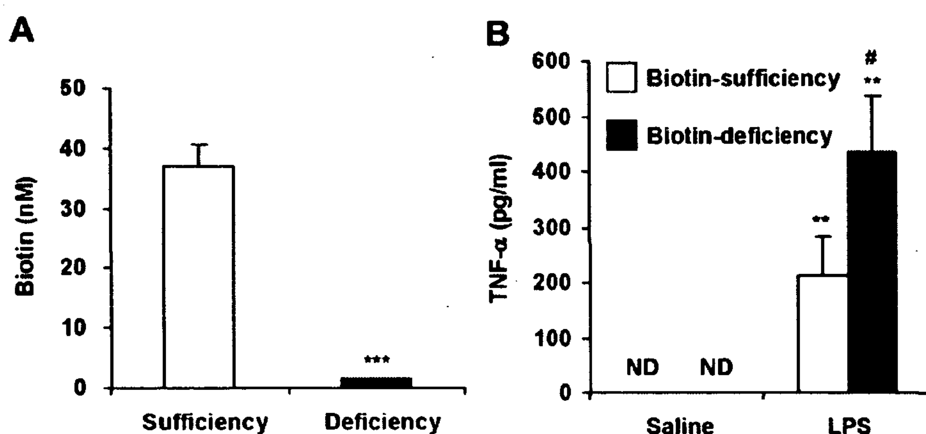

Fig. 1. Serum levels of biotin and TNF-α in biotin-sufficient and -deficient mice. Female BALB/c mice (4 weeks old) were fed a biotin-sufficient or -deficient diet for 8 weeks. (A) Concentrations of biotin in sera were measured by ELISA after prior separation by HPLC. The results were expressed as mean ± SD of triplicate assay. ***, $P < 0.001$, compared with biotin sufficiency. (B) The mice were challenged i.v. with LPS (1 μg/kg) or saline alone, and blood was taken at 90 min after injection. Concentrations of TNF-α in sera were measured by ELISA. The results were expressed as mean ± SD for four mice. ND, Not detected; **, $P < 0.01$, compared with saline; #, $P < 0.05$, compared with biotin sufficiency.