

Promotion of arthritis and allergy in mice by aminoglycoglycerophospholipid, a membrane antigen specific to *Mycoplasma fermentans*

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Abstract

Mycoplasmas, which lack a cell wall and are the smallest self-replicating bacteria, have been linked to some chronic diseases, such as AIDS, rheumatoid arthritis (RA), and oncogenic transformation of cells. Their membrane components (lipoproteins and glycolipids) have been identified as possible causative factors in such diseases. Glycoglycerophospholipid (GGPL)-III, a unique phosphocholine-containing aminoglycoglycerophospholipid, is a major specific antigen of *Mycoplasma fermentans*, and has been detected in 38% of RA patients. Unlike those of lipoproteins, which induce inflammation via Toll-like receptor 2 (TLR2), the pathologic effects of GGPL-III are poorly understood. RA and metal allergies are chronic inflammatory diseases in which autoantigens have been implicated. Here, we examined the effects of chemically synthesized GGPL-III in murine arthritis and allergy models. GGPL-III alone exhibited little inflammatory effect, but promoted both collagen-induced arthritis and nickel (Ni) allergy, although less powerfully than *Escherichia coli* lipopolysaccharide. The augmenting effect of GGPL-III on Ni allergy was present in mice deficient in either T cells or active TLR4, but it was markedly weaker in mice deficient in macrophages, interleukin-1, or the histamine-forming enzyme histidine decarboxylase than in their control strains. These results suggest that GGPL-III may play roles in some types of chronic diseases via the innate immune system.

Introduction

Many (over 190) bacterial species lacking a cell wall are distributed among humans, other vertebrates, insects, and plants. Mycoplasmas (class *Mollicutes*, meaning soft skin) are devoid of a cell wall and are the smallest (diameter 0.2–0.8 µm) self-replicating bacteria. Mycoplasmas have a very small genome (0.58–2.20 Mb, compared with the 4.64 Mb of *Escherichia coli*), and they are thought to have developed from Gram-positive bacteria by genome reduction (Razin *et al.*, 1998). Mycoplasmas adhere to, invade, and fuse with host cells (Razin *et al.*, 1998; Rottem, 2003). They circumvent or modulate the host immune system and are thought to be causally involved in some chronic diseases, such as AIDS progression, rheumatoid arthritis (RA), and

oncogenic transformation of cells (Katseni *et al.*, 1993; Horowitz *et al.*, 2000; Shimizu *et al.*, 2004; Sordet *et al.*, 2005; Kawahito *et al.*, 2008). Some components in their membranes (such as lipoproteins, lipopeptides, and glycolipids) have been identified as possible causative factors in such diseases (Rawadi, 2000; Rottem, 2003; Kawahito *et al.*, 2008). However, whereas lipoproteins or lipopeptides are known to induce inflammatory responses via Toll-like receptors (TLRs) (Seya & Matsumoto, 2002; Akira *et al.*, 2006), little is known about the biological or the pathologic activities of the glycolipids.

During studies on the possible involvement of glycolipids in the unregulated cell growth caused by oncogenic transformation with retrovirus, a Japanese group detected alkaline-labile glycophospholipids in HTLV-I-transformed

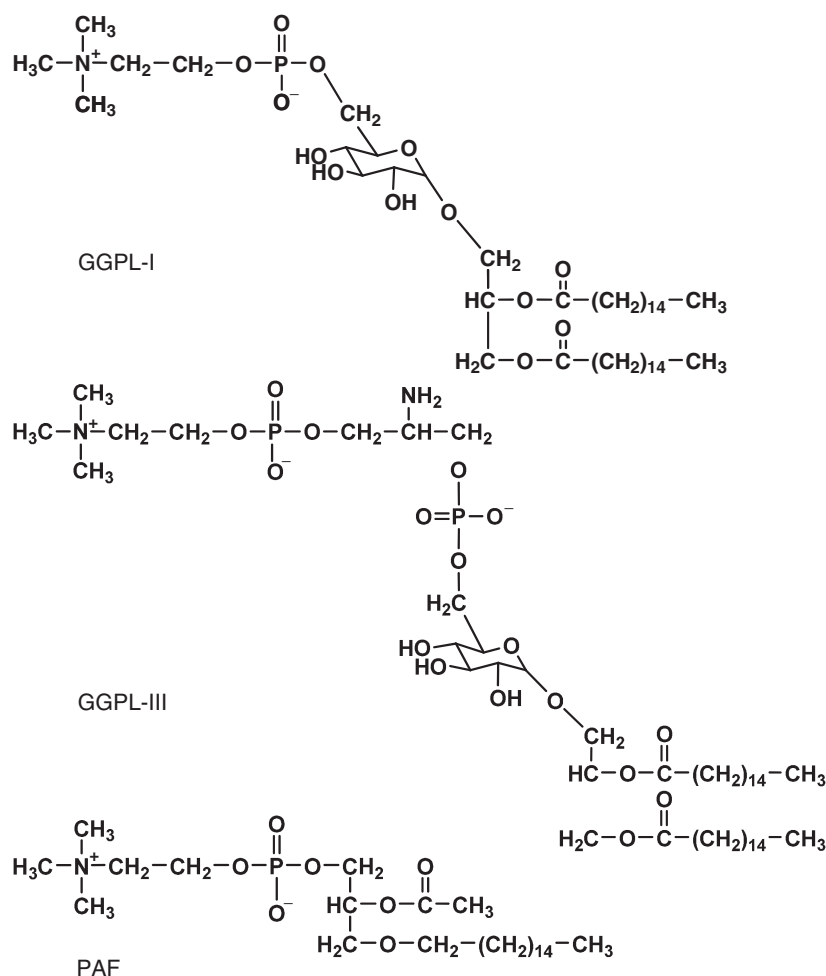


Fig. 1. Structure of GGPL-III and related substances.

T cells (Matsuda *et al.*, 1993). However, they later realized that the presence of these glycolipids resulted from accidental infection with *Mycoplasma fermentans*, and they renamed them glycosylglycerophospholipids (GGPLs) (Matsuda *et al.*, 1995). GGPL-I has a unique structure containing phosphocholine (Matsuda *et al.*, 1994), and GGPL-I and GGPL-III are species-specific major antigens of *M. fermentans* (Li *et al.*, 1997; Matsuda *et al.*, 1997a). GGPL-III, too, has a phosphocholine-containing aminoglycosylglycerolipid structure (Matsuda *et al.*, 1997b; Nishida *et al.*, 1999), and it was synthesized by Nishida *et al.* (1999), who also determined its absolute configuration (Fig. 1). The Japanese group that originally detected GGPLs later obtained a monoclonal antibody that recognizes GGPL-III, but not GGPL-I (Matsuda *et al.*, 2000). In addition to the above studies, an Israeli group, during research on the fusogenic nature of *M. fermentans* (which is capable of fusing with a variety of cells), noted that a lipid component acts as a fusogen (Salman *et al.*, 1994). The structure of this lipid, which they called *M. fermentans* glycolipid (MfGL)-II (Zähringer *et al.*, 1997), was identical to that of GGPL-III. Although purified

MfGL-II induced some inflammatory responses in rat astrocytes, the reproducibility of the response differed among batches of preparations (some preparations showing no activity at all) (Ben-Menachem *et al.*, 1998).

RA and metal allergies are representative chronic inflammatory diseases related to autoantigens or autoantibodies (i.e. adaptive immunity) (Thierse *et al.*, 2005; Brennan & McInnes, 2008; Cope, 2008). Type II collagen emulsified in Freund's complete adjuvant (FCA) is used to immunize DBA/1 mice when inducing experimental collagen-induced arthritis (CIA) (Abdollahi-Roodsaz *et al.*, 2007). In this model, TLR4 or lipopolysaccharide (LPS) is implicated as a promoter of CIA (Dinser, 2008). Further, various bacterial and viral components have been shown to modify adaptive immunity via TLRs (Kaisho & Akira, 2002). Indeed, a peptidoglycan (a TLR2 ligand) obtained from *Staphylococcus epidermidis* is also known to promote CIA (Nakamura *et al.*, 1996). In metal allergies, complexes of self-proteins and metal ions become allergens or autoantigens. We recently reported that a LPS obtained from *E. coli* strongly promotes the establishment of metal allergies in mice via

TLR4 (Sato *et al.*, 2007). Thus, a reasonable supposition might be that like CIA, metal allergies can be promoted by other microbial components, possibly including GGPL-III.

On the basis of the background described above, we hypothesized that GGPL-III might be involved in the development of CIA and nickel (Ni) allergy in mice, and we performed the present study to examine this idea.

Materials and methods

Animals

DBA/1 mice and BALB/c nude mice were purchased from SLC (Shizuoka, Japan). BALB/c mice, interleukin (IL)-1-KO (deficient in both IL-1 α and IL-1 β), and tumor necrosis factor (TNF)- α -KO BALB/c mice (established by Horai *et al.*, 1998), C57BL/6 mice, and HDC-KO C57BL/6 mice (established by Ohtsu *et al.*, 2001) were raised in our laboratory. Female mice (7–9 weeks old) were used in the present study. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

Reagents

GGPL-III was synthesized by Nishida *et al.* (1999). *Escherichia coli* O55:B5 LPS (prepared by phenol extraction) and clodronate (dichloromethylene diphosphonate) were purchased from Sigma (St. Louis, MO). NiCl₂ and FCA or Freund's incomplete adjuvant (FIA) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Bovine type II collagen prepared by the Collagen Research Center (Kiyose, Japan) was purchased from Cosmo-Bio Inc. (Tokyo, Japan). It was dissolved in 0.01 M acetic acid at a concentration of 3 mg mL⁻¹. LPS, NiCl₂, and GGPL-III were dissolved in sterile saline.

Induction of CIA

The precise experimental protocols used to induce arthritis in DBA/1 mice are described in the relevant sections of the text or in the legend to the figure relating to each experiment. The severity of the joint inflammation in each mouse was quantified by scoring each paw on a scale of 0–4 (Wood *et al.*, 1969), the total maximum score for a given mouse (four paws) being 16. The scoring of the inflammation was as follows: 0, no symptoms; 1, redness in fingers, without clear swelling; 2, clear swelling in the fingers; 3, both swelling in the fingers and redness in the palmar manus; and 4, clear or severe swelling of the palmar manus, making the fingers look shorter. Two observers allocated scores in a blinded manner. For convenience, the mean and SD of the total score for a given group were used as indices of the induced arthritis, with statistical analysis of the scores being performed using a nonparametric method.

Induction of Ni allergy

An equivolume mixture of 1 mM NiCl₂ solution and a solution containing a test substance was injected intraperitoneally into mice (0.25 mL per mouse). At 10 days after this sensitizing injection, an equivolume mixture of 5 mM NiCl₂ solution and a solution containing a test substance was given as a challenge by an intradermal injection into the left and right pinnae near the root of the ear (20 μ L each ear). Saline was injected intradermally (20 μ L) as a control for the challenging injection. However, because this saline injection induced no significant ear swelling, these data are not shown in some figures. Mice were anesthetized with ethyl ether just before the challenging injection. Ear swelling was measured at a site 2–3 mm distant from the challenge site at the indicated times [using a Peacock dial thickness gauge (Ozaki Mfg Co. Ltd, Tokyo, Japan)], and the induced difference (after vs. before the challenge) was recorded.

Depletion and detection of macrophages

Clodronate-encapsulated liposomes (Clo-lip) selectively deplete phagocytic macrophages (Van Rooijen & Sanders, 1994). As in our previous studies (Endo *et al.*, 1995; Yamaguchi *et al.*, 2006), a suspension of Clo-lip was intravenously injected into mice, and depletion of macrophages was confirmed by immunostaining of the liver using the F4/80 antibody (Yamaguchi *et al.*, 2006).

Statistical analysis

All experimental values are given as the mean $\pm$ SD. The statistical significance of differences was analyzed using an ANOVA, with a Bonferroni multiple-comparison test performed *post hoc* (INSTAT software). *P*-values < 0.01 were considered to indicate significance. The difference between two experimental groups in terms of the score allocated for arthritis was analyzed using a Ridit (relative to an identified distribution) test (Bross, 1958).

Results

Effects of GGPL-III on CIA

GGPL-III plus FIA promotes CIA

FCA is widely used to promote or enhance various immune responses, including CIA in DBA/1 mice. FCA is a mixture of FIA and mycobacterial cell-wall components, which are thought to promote cell-mediated immune responses. Because a peptidoglycan unit of *S. epidermidis* also promotes CIA (Nakamura *et al.*, 1996), we hypothesized that GGPL-III might also promote CIA. To test this hypothesis, we evaluated the effect of FIA+GGPL-III on CIA, using FCA as a positive control.

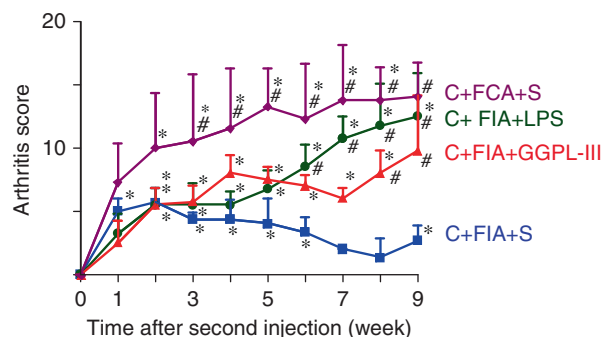


Fig. 2. Adjuvant activity of GGPL-III+FIA in the induction of collagen arthritis in DBA/1 mice. Bovine type II collagen [3 mg mL^{-1} in 0.01 M acetic acid (abbreviated as C)] was used as an antigen to induce arthritis. The following mixtures (4 : 5 : 1 by volume, in the order mentioned) were prepared: (1) C + FIA + S, (2) C + FCA + S, (3) C + FIA + LPS ($1 \mu\text{g mL}^{-1}$), and (4) C + FIA + GGPL-III (5 mg mL^{-1}). All mixtures were homogeneously emulsified using a sonicator. Each mixture ($50 \mu\text{L}$) was injected intradermally at the root of the tail in DBA/1 mice (first immunization). Three weeks later, the same mixture was injected again in the same way (second immunization). Arthritis scores were recorded each week after the second immunization. Each value is the mean $\pm$ SD from five mice. $^{\#}P < 0.01$ vs. time 0, $^{*}P < 0.01$ vs. C + FIA + S.

As shown in Fig. 2, FCA strongly promoted CIA [see C + FCA + saline (S) group], while the effect of FIA (see C + FIA + S) was weak and transient. As we had hypothesized, GGPL-III clearly promoted CIA, although its effect was weaker than that of LPS. These results suggest that when given together with FIA, GGPL-III has adjuvant activity, inducing CIA.

GGPL-III alone (without FIA) has little ability to promote CIA

Next, we examined whether administering GGPL-III by itself (i.e. without FIA) might promote CIA. In this experiment, too, FCA was used as a positive control. As shown in Fig. 3, type II collagen alone (see C + S group) increased the arthritis score slightly and transiently. LPS tended to hasten the onset of CIA, but even so its effect was slight and transient. There was no detectable difference between the effects of C + GGPL-III and C + S, suggesting that by itself, GGPL-III has little or no ability to promote CIA.

Effects of GGPL-III on Ni allergy

GGPL-III alone has little inflammatory effect

As shown in Fig. 4a, GGPL-III (0.04 , 0.2 , or 1 mg mL^{-1}), when injected into the ear pinnae of BALB/c mice, induced no detectable swelling, suggesting that its inflammatory effect is very small or nonexistent.

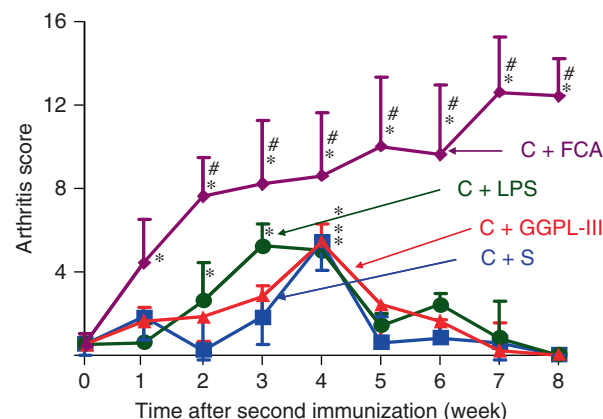


Fig. 3. Adjuvant activity of GGPL-III alone in the induction of collagen arthritis in DBA/1 mice. The following mixtures (1 : 1 by volume) were prepared: (1) C (see legend to Fig. 2) + S, (2) C + LPS ($1 \mu\text{g mL}^{-1}$), (3) C + GGPL-III (1 mg mL^{-1}), and (4) C + FCA. Mixture (4) was homogeneously emulsified using a sonicator. Each mixture ($50 \mu\text{L}$) was injected intradermally at the root of the tail in DBA/1 mice (first immunization). Three weeks later, the same mixture was injected again in the same way (second immunization). Arthritis scores were recorded each week after the second immunization. Each value is the mean $\pm$ SD from five mice. $^{\#}P < 0.01$ vs. time 0, $^{*}P < 0.01$ vs. C + S.

GGPL-III alone has little antigenic effect

In experimental models of allergies, an antigen is administered to a given animal twice, with an interval, to sensitize it to the antigen and then to elicit allergic inflammation. Here, we tested whether GGPL-III has an antigenic effect in BALB/c mice. An intraperitoneal injection (0.25 mL per mouse) of an equivolume mixture of GGPL-III (1 mg mL^{-1}) and saline, followed (10 days later) by an injection of GGPL-III (1 mg mL^{-1}) into the ear pinnae ($20 \mu\text{L}$ per ear) produced no inflammatory swelling in the ear (Fig. 4b). This result suggests that GGPL-III has little antigenicity under the present experimental conditions. We reported previously that LPS promotes sensitization to (or enhances the antigenicity of) metals or ovalbumin (Sato *et al.*, 2007; Yoshida *et al.*, 2007). We therefore examined whether LPS had the ability to sensitize BALB/c mice to GGPL-III. As shown in Fig. 4b, GGPL-III injection into the ear pinnae of mice pretreated with GGPL-III + LPS induced no detectable ear swelling. This suggests that GGPL-III has little antigenicity even when injected together with LPS at the sensitization step, and that by itself, GGPL-III exhibits little, if any, antigenicity in BALB/c mice.

GGPL-III promotes Ni allergy at both the sensitization and the elicitation steps

As described in Introduction, LPS promotes Ni allergy both at the sensitization step and at the elicitation step (Sato

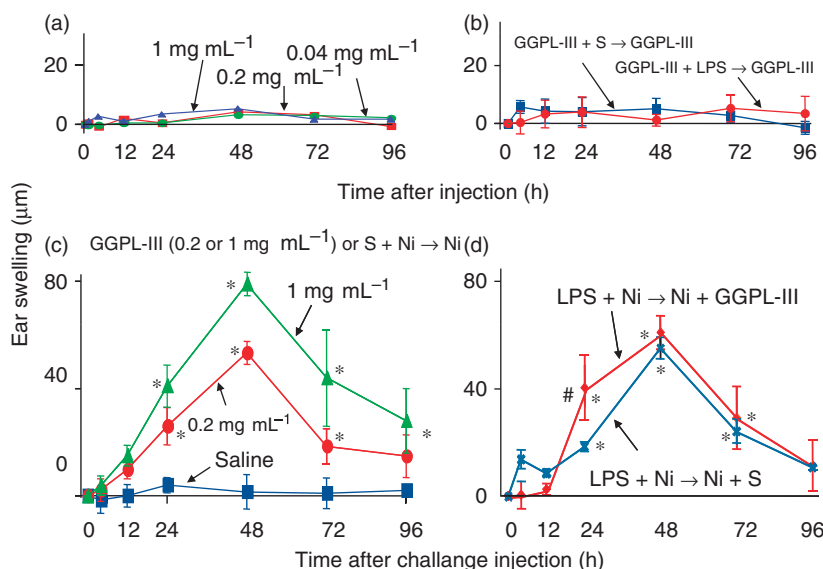


Fig. 4. Effects of GGPL-III on Ni allergy in BALB/c mice. (a) By itself, GGPL-III induced no detectable inflammation. GGPL-III solution (0.04, 0.2, or 1 mg mL⁻¹) was injected into the ear pinnas (20 μL per ear), and ear swelling was measured thereafter. (b) By itself, GGPL-III was not allergenic. A mixture (1 : 1 by volume) of GGPL-III (1 mg mL⁻¹) + S or GGPL-III (1 mg mL⁻¹) + LPS (1 μg mL⁻¹) was injected intraperitoneally (0.25 mL per mouse). Ten days later, GGPL-III (1 mg mL⁻¹) was injected into the ear pinnas (20 μL per ear), and ear swelling was measured at intervals thereafter. (c) GGPL-III promoted Ni allergy at the sensitization step. A mixture (1 : 1 by volume) of GGPL-III (0.2 or 1 mg mL⁻¹) + NiCl₂ (1 mM) or S + NiCl₂ (1 mM) was injected intraperitoneally into mice (0.25 mL per mouse). Ten days later, NiCl₂ (5 mM) was injected into the ear pinnas (20 μL per ear) (i.e. challenge injection), and ear swelling was measured at intervals thereafter. (d) GGPL-III promoted Ni allergy at the elicitation step, too. A mixture (1 : 1 by volume) of LPS (1 μg mL⁻¹) + NiCl₂ (1 mM) was injected intraperitoneally into mice (0.25 mL per mouse). Ten days later, a mixture (1 : 1 by volume) of NiCl₂ (5 mM) + GGPL-III (1 mg mL⁻¹) or NiCl₂ (5 mM) + S was injected into the ear pinnas (20 μL per ear) (i.e. challenge injection), and ear swelling was measured at intervals thereafter. Each value is the mean ± SD from three to four mice (i.e. *n* = 6–8 ears). **P* < 0.01 vs. time 0, #*P* < 0.01 vs. LPS + Ni → Ni + LPS.

et al., 2007). We therefore examined whether GGPL-III also exerts such effects. As shown in Fig. 4c, the injection of GGPL-III into the ear pinnas of mice pretreated with GGPL-III + NiCl₂ induced marked ear swelling in a manner related to the concentration used at the sensitization step. In the next experiment, mice were sensitized to Ni by an intraperitoneal injection of LPS + NiCl₂, and 10 days later, their ears were injected with a mixture of NiCl₂ + GGPL-III or NiCl₂ + S. As shown in Fig. 4d, the swelling detected at 24 h was significantly greater in the group given NiCl₂ + GGPL-III than in that given NiCl₂ + S. These results suggest that GGPL-III (like LPS) promotes Ni allergy regardless of whether it is given at the sensitization step or at the elicitation step.

Adjuvant activity of GGPL-III in Ni allergy is dependent on innate immunity

In the following experiments, mice were sensitized to Ni by an intraperitoneal injection of an equivolume mixture of GGPL-III (1 mg mL⁻¹) + NiCl₂ (1 mM), and 10 days later, their ear pinnas were challenged with NiCl₂ (5 mM). This allergy is hereafter termed 'Ni(+GGPL-III) allergy'. We

obtained the following results: (1) Ni(+GGPL-III) allergy was induced in nude BALB/c mice deficient in T cells (Sato *et al.*, 2007) (Fig. 5a), suggesting that this allergy is inducible in the absence of T cells. (2) Ni(+GGPL-III) allergy was significantly weaker in C3H/HeJ mice (with mutated TLR4) than in control C3H/HeN mice, suggesting that the involvement of TLR4 in this allergy is limited (Fig. 5b). (3) Ni(+GGPL-III) allergy was very weak in Clo-lip-treated (i.e. macrophage-depleted) mice (Fig. 5c), suggesting that macrophages may play an important role in this allergy. (4) IL-1-KO mice, but not TNF-α-KO mice, were resistant to Ni(+GGPL-III) allergy (Fig. 5d and e), suggesting that IL-1 is involved in this allergy. (5) Ni(+GGPL-III) allergy was markedly weaker in mice deficient in the histamine-forming enzyme histidine decarboxylase (HDC-KO mice) than in the control wild-type C57BL/6 mice (Fig. 5f), suggesting that HDC (or HDC-induced histamine) is involved in this allergy.

Discussion

In the present study, we examined the effects of chemically synthesized GGPL-III in murine arthritis and allergy

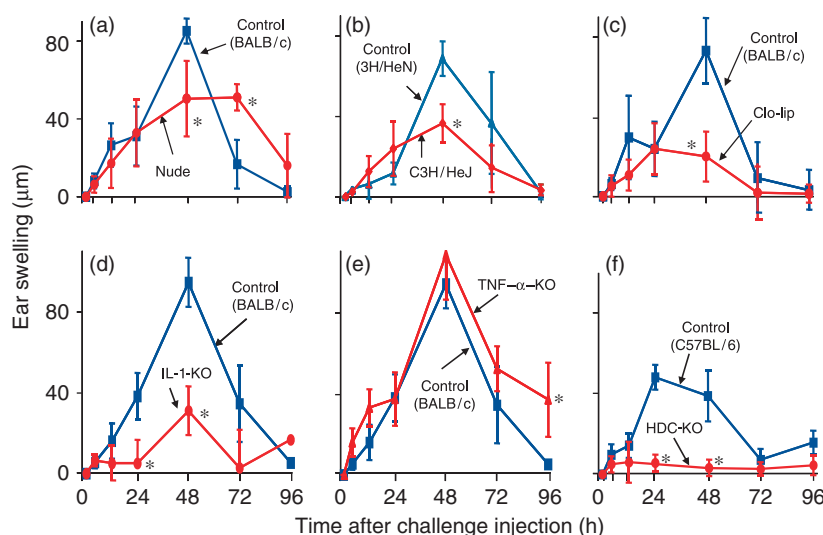


Fig. 5. Induction of Ni(+GGPL-III) allergy in various KO or mutant mice. (a) Nude (T cell-deficient) and control BALB/c mice, (b) C3H/HeJ (TLR4-mutant) and control C3H/HeN mice, (c) macrophage-depleted and control (saline-injected instead of Clo-lip-injected) BALB/c mice, (d) IL-1-KO mice and control BALB/c mice, (e) TNF- α -KO mice and control BALB/c mice, and (f) histidine decarboxylase (HDC, the histamine-forming enzyme)-KO mice and control C57BL/6 mice. The mice were intraperitoneally injected with a mixture (1 : 1 by volume) of GGPL-III (1 mg mL⁻¹) + NiCl₂ (1 mM) (0.25 mL per mouse). Ten days later, NiCl₂ (5 mM) was injected into the ear pinnae (20 μ L per ear) (i.e. challenge injection), and ear swelling was measured at intervals thereafter. In C, the GGPL-III + NiCl₂ mixture was given 24 h after the injection of Clo-lip or saline. Each value is the mean $\pm$ SD from four mice (i.e. $n = 8$ ears). * $P < 0.01$ vs. control.

models. By itself, GGPL-III displayed little inflammatory effect, but it promoted both CIA and Ni allergy (although its effects were much weaker than those of *E. coli* LPS). The augmenting effect of GGPL-III on Ni allergy was seen in mice deficient either in T cells or in active TLR4, but it was markedly weaker in mice deficient in macrophages, IL-1, or the histamine-forming enzyme HDC than in their control strains. In the following paragraphs, we discuss these effects of GGPL-III.

Mycoplasma fermentans is reportedly present in synovial fluid obtained from RA patients (Schaeffer *et al.*, 1996a, b; Haier *et al.*, 1999; Horowitz *et al.*, 2000; Gilroy *et al.*, 2001). GGPL-III is a major, specific antigen of *M. fermentans*, and Kawahito *et al.* (2008) recently reported detecting it in synovial tissues obtained from 38% (32/84) of RA patients, but not in those from osteoarthritic ($n = 20$) or normal ($n = 10$) joints. Thus, the present results may support the idea that in RA, GGPL-III or *M. fermentans* might be a causal or an exacerbating factor. If so, administration of antimycoplasma agents to GGPL-III-positive patients might be effective as a therapeutic strategy, at least in the early stages of RA.

On the grounds that lipoproteins by themselves are inflammatory, whereas GGPL-III by itself is not, it might be considered that lipoproteins are involved in RA, and that they are more important than GGPL-III. However, we think it noteworthy that GGPL-III, even if it has little inflammatory effect by itself, exhibits RA- and allergy-promoting effects. If lipoproteins are indeed major pathogenic substances of mycoplasmas, acute inflammation might be expected to be caused by these bacteria. In fact, mycoplasmas are mainly implicated in chronic inflammatory diseases. Hence, we speculate that the acute inflammatory effects of the lipoproteins of mycoplasmas (i.e. stimulation

of TLRs) may be masked in some way, enabling these bacteria to circumvent the host immune system (Rottem, 2003). On the other hand, GGPL-III may play important roles in chronic inflammatory diseases.

Various substances of bacterial origin, such as peptidoglycan (a TLR2 ligand) and CpG-containing DNA (a TLR9 ligand), have been found in the synovial fluid of RA patients (van der Heijden *et al.*, 2000). We showed previously that a peptidoglycan from *S. epidermidis* promotes CIA in DBA/1 mice (Nakamura *et al.*, 1996). In the present study, GGPL-III, when emulsified with FIA, was found to promote CIA, although its effect was weaker than that of LPS (a TLR4 ligand). These findings support the idea that bacterial components, including GGPL-III, may be causally involved in RA via the innate immune system.

Unlike the classical haptens, metal ions form reversible coordination complexes with partner molecules. It is therefore difficult to identify the allergenic epitopes of such reversible complexes, and the host may recognize metal ions in complicated ways (Thierse *et al.*, 2005). Because the partner molecules that confer allergenicity on metals are self-proteins, we speculate that metal allergies might belong to the autoimmune diseases. As described in Introduction, LPS potently promotes Ni allergy, which we therefore tentatively call 'Ni(+LPS) allergy'. Like Ni(+LPS) allergy, Ni(+GGPL-III) allergy was inducible in nude mice, and our results indicate that macrophages, IL-1, and HDC are involved in the latter type of allergy. The roles of these factors in Ni(+LPS) allergy have been discussed by us in a previous paper (Sato *et al.*, 2007). As far as we can tell, the only difference between these Ni allergies may be that Ni(+GGPL-III) allergy can be induced in C3H/HeJ mice, a TLR4-mutant strain (the present study), whereas Ni(+LPS) allergy cannot (Sato *et al.*, 2007). However, we should point

out that the Ni(+GGPL-III) allergy in C3H/HeJ mice was somewhat weaker than that observed in control C3H/HeN mice (Fig. 5b).

Histamine regulates immune responses in association with Th1 and Th2 cells (Jutel *et al.*, 2002; Schneider *et al.*, 2002), and it is known that a number of inflammatory agents (including LPS) induce HDC (the histamine-forming enzyme) in various tissues in mice (Endo, 1983; Wu *et al.*, 2004). The process by which HDC is induced is very sensitive to LPS. Indeed, an intravenous injection of as little as $0.25 \mu\text{g kg}^{-1}$ (c. 5 ng per mouse) of *E. coli* LPS induces HDC (Endo, 1982), and C3H/HeJ mice are highly resistant to the induction of HDC by LPS (Endo, 1983), indicating that HDC is sensitively induced via stimulation of TLR4. GGPL-III, however, did not induce HDC in BALB/c mice (data not shown), suggesting that GGPL-III may not itself directly stimulate TLR4. We recently found that agonists of nucleotide-binding oligomerization domain-like receptors augment the induction of HDC by various TLR agonists (Funayama *et al.*, 2009). Interestingly, it has been suggested that TLR4 is stimulated by endogenous ligands (Tsan & Gao, 2004; Wagner, 2006). Thus, it seems likely that GGPL-III acts as a primer for the effects of endogenous TLR4 agonists and/or that it interacts with endogenous TLR4 agonists, resulting in TLR4-stimulating effects. However, many experiments would be needed to test this attractive hypothesis.

In the present study, we examined the *in vivo* inflammatory effects of chemically synthesized GGPL-III. Various bacterial inflammatory agents are known to induce proinflammatory cytokines via TLRs (Kaisho & Akira, 2002, 2004). In addition, because GGPL-III is structurally similar to platelet-activating factor (PAF) (Matsuda *et al.*, 2000) (Fig. 1), and because some LPS preparations induce an accumulation of platelets in the lung and/or the liver in mice (Endo *et al.*, 1997; Zhao *et al.*, 2002), we also examined the effect of GGPL-III on the production of proinflammatory cytokines and on platelet behaviors *in vivo*. However, neither an intravenous nor an intraperitoneal injection of GGPL-III into BALB/c mice produced any detectable elevations of the serum levels of IL-1 β and TNF- α or any detectable changes in platelet behaviors (data not shown). These results, together with the lack of effect on HDC described above, suggest that as regards the induction of acute inflammation, the activity of GGPL-III by itself is very weak, which may also be a characteristic of *M. fermentans*.

In conclusion, GGPL-III and/or *M. fermentans* may play roles in some types of chronic inflammatory diseases via the innate immune system.

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