

A TLR2 Ligand Suppresses Allergic Inflammatory Reactions by Acting Directly on Mast Cells

Kazumi Kasakura Kyoko Takahashi Tomoyuki Aizawa Akira Hosono
Shuichi Kaminogawa

Laboratory of Biological Chemistry on Food Functionalities, Department of Food Science and Technology,
College of Bioresource Sciences, Nihon University, Kanagawa, Japan

Key Words

Mast cell • Toll-like receptor • Probiotic • *Bifidobacterium* • Degranulation • Cytokine • Leukotriene • Vascular permeability • Intracellular signal

Abstract

Background: Although much attention has been focused on the anti-allergic effects of probiotics, their mode of action is not fully understood. Mast cells, which play a central role in inducing allergic inflammation, are potential targets of probiotics given the recent discovery that they express Toll-like receptors (TLRs), the pattern recognition receptors for microbial components. In this study, we examined whether allergic reactions of mast cells are modulated by stimulation through TLR2. **Methods:** The effects on mast cells of the synthetic TLR2 ligand Pam3CSK4 and *Bifidobacterium pseudocatenulatum* JCM 7041 were evaluated in vitro. Furthermore, the effects of Pam3CSK4 on mast cell-induced increase in vascular permeability in vivo were investigated by employing mast cell-deficient W/W^v mice into which IgE-sensitized mouse bone marrow-derived mast cells were transferred. **Results:** Pam3CSK4 and *Bifidobacterium pseudocatenulatum* JCM 7041 suppressed degranulation of IgE-sensitized mast cells upon antigen stimulation in vitro. Pam3CSK4 also suppressed leukotriene C₄ production triggered by engagement of the high-affinity IgE receptor,

FcεRI. Intracellular Ca²⁺ mobilization and phosphorylation of Erk were suppressed by pretreatment with Pam3CSK4, suggesting that the TLR2 ligand suppresses activation of mast cells by interrupting FcεRI-mediated intracellular signaling. Pam3CSK4 treatment of bone marrow-derived mast cells reduced the increase in vascular permeability in recipient W/W^v mice upon intravenous injection of antigen; the decrease was by about half, in a TLR-dependent manner. **Conclusion:** Collectively, these results demonstrate that the FcεRI-mediated inflammatory responses of mast cells are suppressed by stimulation through TLR2, suggesting that probiotics exert potential anti-allergic effects, at least in part, through direct effects on mast cells.

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Introduction

It is a serious social problem that a large and increasing population suffers from some form of allergy. The potential thus exists for foods – including probiotics such as *Lactobacillus* and *Bifidobacterium* – defined as live microorganisms that confer health benefits on the host to prevent or alleviate the symptoms of allergy. It has been proposed in several large-scale randomized double-blind controlled trials [1–3] that probiotic microbes prevent atopic eczema in infants. However, unequivocal evidence

has not been provided to demonstrate the suppression of allergy by probiotics probably because of the complicated pathophysiology of allergy, differences in bacterial strains employed for each experiment, and the mildness of the effects. In spite of numerous reports on the anti-allergic effects of probiotics, their mode of action is not yet fully elucidated. In animal studies, a decrease in serum IgE levels supported by an improvement in the Th1/Th2 balance and/or induction of regulatory T cells is generally believed to be associated with the suppression of allergy by probiotics [4–6]. However, clinical symptoms are often alleviated without a decrease in serum IgE levels in human trials, suggesting that different mechanisms independent of IgE sensitization also work.

Mast cells, which express the high-affinity IgE receptor (FcεRI) on their surface, play an essential role in inducing allergic inflammation in peripheral tissues. Cross-linking of FcεRI on mast cells by antigen (Ag)-IgE complexes triggers an allergic reaction by activating intracellular signal cascades to induce not only the release of chemical mediators, including histamine, in the early-phase reaction, but also Th2 and inflammatory cytokine production leading to the late-phase reaction. Recently, in addition to their role as effector cells in allergy, the protective role of mast cells during acute infection has been featured as their primary physiological function [7–10]. Furthermore, recent reports have revealed that mast cells express various Toll-like receptors (TLRs), the pattern recognition receptors for microbial components, and are activated through the receptors [11–14], raising the possibility that microbial components directly influence the functions of mast cells.

The effects of orally administered probiotics are thought to be exerted through influences on both intestinal microbiota and the intestinal immune system. Among the various types of cells constituting the intestinal immune system, mast cells, in addition to Ag-presenting cells and epithelial cells, may be a potential target of probiotics. In this study, to elucidate one of the underlying mechanisms behind the suppressive effects of probiotics on allergy, we examine whether stimulation of mast cells through TLR2, which is known to mainly recognize cell wall components of Gram-positive bacteria, modulates their allergic reactions, because most probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* belong to the Gram-positive group. Elucidation of the influences of stimulation through TLR2 on the allergic reactions of mast cells will help us to optimize probiotic use for anti-allergy purposes.

Methods

Mice

C57BL/6 mice, WBB6F1-W/W^v mice and MyD88^{-/-} mice on a C57BL/6 background were purchased from CLEA Japan (Tokyo, Japan), Japan SLC (Hamamatsu, Japan) and Oriental BioService (Kyoto, Japan), respectively. All animal experiments were carried out in accordance with the guidelines for care and use of laboratory animals of Nihon University.

Cell Culture

Rat basophilic leukemia (RBL)-2H3 cells were cultured in Eagle's MEM supplemented with 10% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 5×10^{-5} M 2-mercaptoethanol at 37°C in a humidified incubator with 5% CO₂. Mouse bone marrow-derived mast cells (BMMCs) were generated as follows. Bone marrow cells were prepared from femurs of 7-week-old female wild type (WT) or MyD88^{-/-} C57BL/6 mice and cultured in RPMI supplemented with 10% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine, 0.1 mM nonessential amino acids, 100 µg/ml sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and 5 ng/ml recombinant mouse IL-3. Cells were used for the experiments as BMMCs after 4–6 weeks of culture. The c-kit and FcεRI double-positive phenotype of the BMMCs obtained was confirmed by flow cytometric analyses with PE-Cy7-labeled anti-c-kit Ab (eBioscience, San Diego, Calif., USA) and Alexa 488-labeled IgE.

Preparation of Bacterial Components

Bifidobacterium pseudocatenulatum JCM 7041 (Bp) was obtained from the Japan Collection of Microorganisms of RIKEN Bioresource Center (Saitama, Japan). Sonicated components of Bp were prepared as previously described [15]. Briefly, Bp was cultured in GAM broth at 37°C under anaerobic conditions. After washing and freeze drying, Bp cells were ultrasonically disrupted with the aid of a Branson Sonifier 450 (Branson, Danbury, Conn., USA) for 15 min. The supernatant separated by centrifugation at 800 g for 10 min was freeze dried and employed for experiments.

Degranulation and Leukotriene C₄ Production

RBL-2H3 cells were incubated overnight at 1.25×10^5 cells/well in a 24-well plate. Alternatively, BMMCs from WT or MyD88^{-/-} C57BL/6 mice were incubated at 2×10^5 cells/well in a 96-well plate. Cells were then sensitized with 0.2 µg/ml of TNP-specific mouse IgE (clone 15.3.2, BD Bioscience Pharmingen, San Diego, Calif., USA) for 2 h in the presence or absence of sonicated Bp or the synthetic lipoprotein Pam3CSK4 (Invivogen, San Diego, Calif., USA). After washing with Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% bovine serum albumin), cells were stimulated with TNP-bovine serum albumin (LSL, Tokyo, Japan) for 30 min prior to collection of the culture supernatants. For determination of degranulation, the release of β-hexosaminidase was measured as follows. Culture supernatants were incubated with 2.67 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Nacalai Tesque, Kyoto, Japan) for 40 min at 37°C. After developing the reaction with 0.2 M glycine (pH 11), absorbance was measured at 450 nm. Release was calculated as a percentage to the total β-hexosaminidase content determined by cell lysis with

0.1% Triton-X 100. Concentrations of leukotriene C_4 (LTC $_4$) in the culture supernatant were determined using the Leukotriene C_4 EIA Kit (Cayman Chemical, Ann Arbor, Mich., USA), according to the manufacturer's instructions.

FcεRI Expression and IgE Binding

Mouse IgE (clone 15.3.2) was labeled with Alexa 488 using a labeling kit purchased from Invitrogen (Carlsbad, Calif., USA). RBL-2H3 cells were cultured overnight at 5×10^5 cells/well in a 6-well plate. To analyze the effects of Bp and Pam3CSK4 on the cell surface expression of FcεRI, cells were incubated for 2 h in the medium at 37°C in a CO $_2$ incubator with or without sonicated Bp (1,000 μg/ml) or Pam3CSK4 (10 μg/ml). After washing with Hanks' balanced salt solution, cells were incubated with Alexa 488-labeled IgE on ice for 30 min and analyzed by flow cytometry. To analyze the effects on IgE binding to cell surface FcεRI, cells were suspended in DMEM (pH 7.2) containing 0.5% FACS bovine serum albumin, 1 mM EDTA, 10 mM HEPES, 2 mM sodium pyruvate and 0.02% sodium azide and incubated with Alexa 488-labeled IgE on ice for 2 h in the presence or absence of Bp (1,000 μg/ml) or Pam3CSK4 (10 μg/ml) prior to flow cytometry analyses.

Cytokine Production

RBL-2H3 cells were incubated overnight at 1.25×10^5 cells/well in a 24-well plate. After treatment with 0.2 μg/ml IgE and 0–10 μg/ml Pam3CSK4 for 2 h, cells were washed twice with MEM and stimulated with Ag (30 ng/ml) for 3 h (for tumor necrosis factor-α, TNF-α) or 6 h (for IL-13). Concentrations of TNF-α and IL-13 in the culture supernatants were quantified by immunoassay kits (BioSource, Camarillo, Calif., USA) according to the manufacturer's instructions.

Cell Viability

RBL-2H3 cells were incubated overnight at 5×10^5 cells/well in a 6-well plate. Cells were treated with or without 10 μg/ml Pam3CSK4 for 2 h, washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM HEPES, pH 7.4, containing 14 mM NaCl $_2$ and 2.5 mM CaCl $_2$) at a concentration of 1×10^6 cells/ml. Then, 1×10^5 cells were stained with 5 μl of annexin V-FITC (BD Bioscience Pharmingen) and 0.04 μg of propidium iodide (Sigma, St. Louis, Mo., USA) for 15 min at room temperature in the dark. After adding 400 μl of binding buffer to each tube, cells were analyzed on a flow cytometer within 1 h.

Western Blotting

RBL-2H3 cells were incubated overnight at 1×10^6 cells/well in a 6-well plate. After sensitizing with 0.2 μg/ml IgE in the presence or absence of 10 μg/ml Pam3CSK4, cells were washed with MEM and then stimulated with 10 ng/ml Ag. After 1, 5, 15 and 30 min, cells were washed with ice-cold PBS and incubated on ice for 30 min in the lysis buffer (20 mM Tris, pH 7.6, 1% Nonidet P-40, 60 mM octyl-β-glucoside, 50 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulphonylfluoride, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin). Proteins were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes for immunoblotting. Antibodies to the following proteins were used as primary antibodies: phosphotyrosine, phospho-extracellular signal-regulated kinase (ERK)1/2, ERK1/2.

Horseradish peroxidase-linked anti-rabbit IgG Ab was used as secondary antibody. The antibodies were all purchased from Cell Signaling Technology (Beverly, Mass., USA).

Intracellular Ca $^{2+}$ Mobilization

RBL-2H3 cells were incubated overnight at 5×10^5 cells/well in a 6-well plate. Cells were sensitized with 0.2 μg/ml IgE in the presence or absence of 10 μg/ml Pam3CSK4 for 2 h, washed twice with Hanks' balanced salt solution and then incubated with 16 μM Fluo 3-AM (Invitrogen) at 37°C for 20 min. Cells were then suspended in Tyrode's buffer and analyzed by flow cytometry. After measuring the baseline concentration of intracellular Ca $^{2+}$ before Ag stimulation, 10 ng/ml Ag was added and the change in intracellular Ca $^{2+}$ concentration measured. In addition, to measure Ca $^{2+}$ release from the endoplasmic reticulum (ER), cells were suspended in Ca $^{2+}$ -free Tyrode's buffer supplemented with 3 mM EGTA (Sigma-Aldrich Japan, Tokyo, Japan) and analyzed similarly. For the measurement of external Ca $^{2+}$ influx, cells were suspended in Ca $^{2+}$ -free Tyrode's buffer supplemented with 3 mM EGTA. After treating the cells with 0.1 μM thapsigargin (Wako Pure Chemical Industries, Osaka, Japan) to deplete the intracellular Ca $^{2+}$ store, Ag was added together with 12 mM CaCl $_2$.

Vascular Permeability

BMMCs from WT or MyD88 $^{-/-}$ C57BL/6 mice were sensitized with 0.5 μg/ml IgE in the presence or absence of 1 μg/ml Pam3CSK4 for 2 h. After washing twice with PBS, 5×10^4 cells/head of BMMCs were injected intradermally into the backs of 8- to 9-week-old female WBB6F1-W/W ^{v} mice. Five to 6 h later, mice were injected intravenously with 200 μg of Ag and 0.5% Evans blue and sacrificed after 10 min. Extravasated Evans blue was extracted with dimethylformamide at 50°C overnight and measured colorimetrically at 570 nm.

Statistical Analysis

Statistical analysis was performed with the 2-tailed Student's *t* test.

Results

We first examined the effects of stimulation through TLR2 on the degranulation of mast cells upon FcεRI engagement using the rat basophilic leukemia cell line RBL-2H3, which is known to express mast cell-specific molecules. RBL-2H3 cells were treated with the synthetic TLR2 ligand Pam3CSK4 or sonicated components of Bp, the Gram-positive intestinal bacterium isolated from human feces, at the time of IgE sensitization and incubated for 2 h until stimulation with Ag. Release of β-hexosaminidase was calculated as a percentage of the total β-hexosaminidase content determined by cell lysis (fig. 1a, c). Although inhibition patterns by Pam3CSK4 or Bp were reproducible for every experiment, standard deviations were large in the graphs because the release induced by stimulation with IgE/Ag in the absence of Pam3CSK4

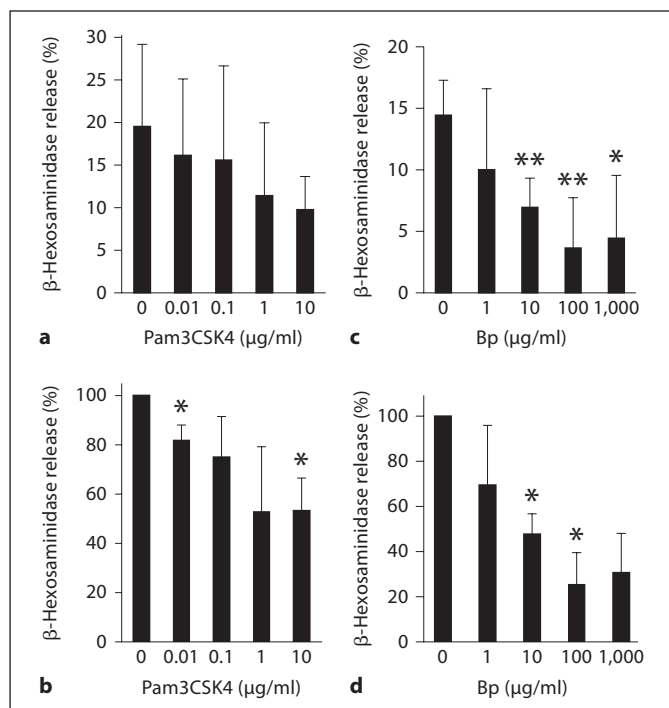


Fig. 1. Effect of Pam3CSK4 and Bp on degranulation of RBL-2H3 cells. RBL-2H3 cells were sensitized with IgE in the presence of the indicated concentrations of Pam3CSK4 (**a, b**) or Bp (**c, d**) for 2 h. After stimulation with 1 or 3 ng/ml of Ag, degranulation was determined by measuring the release of β -hexosaminidase. The release was calculated as a percentage to the total β -hexosaminidase content determined by cell lysis with 0.1% Triton-X 100 (**a, c**). Alternatively, degranulation rates are expressed relative to those of cells stimulated solely with IgE/Ag in the absence of *Bifidobacterium* components or Pam3CSK4 (**b, d**). Results are represented as the means $\pm$ SD of 3–4 independent experiments. * $p < 0.05$, significantly different from the control without Pam3CSK4 treatment; ** $p < 0.01$, significantly different from the control without Bp treatment.

or Bp changed with different experiments (10.6–29.7%) probably as a result of slight variations in the cell culture conditions. To clearly compare the effects of Pam3CSK4 and Bp among these experiments, we expressed the data relative to those of the cells solely stimulated with IgE/Ag in the absence of Pam3CSK4 or Bp (fig. 1b, d). As shown in figure 1b, Pam3CSK4 was revealed to suppress the degranulation of IgE-sensitized RBL-2H3 cells upon Ag stimulation in a dose-dependent manner. In addition, the degranulation of RBL-2H3 cells stimulated with IgE/Ag was shown to be suppressed by sonicated Bp in a dose-dependent manner (fig. 1d). Pam3CSK4 and Bp barely induced the β -hexosaminidase release by themselves, although they induced only slight degranulation at high

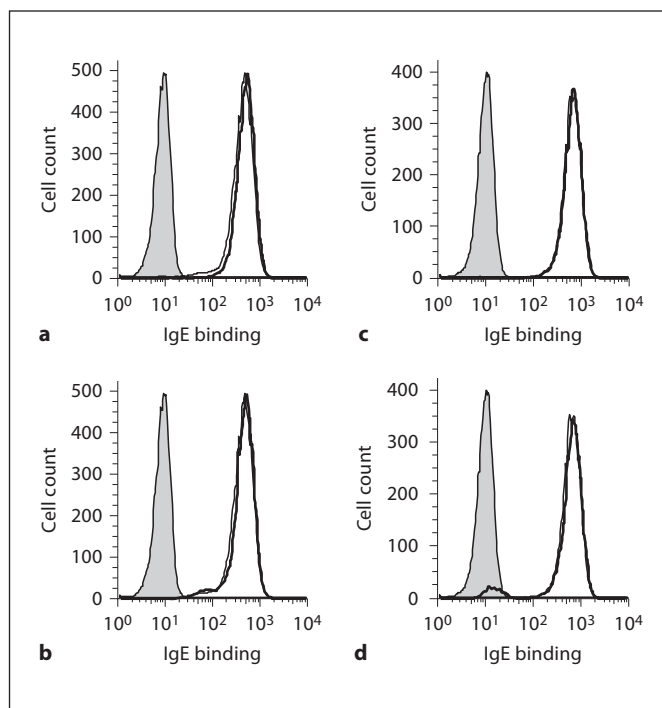


Fig. 2. Effect of Pam3CSK4 and Bp on IgE binding to the surface of RBL-2H3 cells. RBL-2H3 cells were analyzed by flow cytometry to evaluate the influence of Pam3CSK4 (10 μ g/ml; **a, c**) and sonicated Bp (1,000 μ g/ml; **b, d**) on Fc ϵ RI expression (**a, b**) and binding of IgE to cell surface Fc ϵ RI (**c, d**). Cells were cultured for 2 h in medium with or without Pam3CSK4 or Bp. After washing, cells were stained with Alexa 488-labeled IgE (**a, b**). Alternatively, cells were suspended in buffer containing sodium azide and incubated on ice for 2 h with Alexa 488-labeled IgE in the presence or absence of Pam3CSK4 or Bp (**c, d**). Shaded areas = – IgE; thin lines = + IgE without Pam3CSK4 or Bp; bold lines = + IgE with Pam3CSK4 or Bp.

concentrations (data not shown). The suppressive effects seen in figure 1 were not due to the inhibition of IgE binding to the cell surface, because neither cell surface expression of Fc ϵ RI nor binding of IgE to Fc ϵ RI on RBL-2H3 cells was affected by the addition of Pam3CSK4 or sonicated Bp (fig. 2).

We next determined the effects of Pam3CSK4 on the production of cytokines and arachidonic acid metabolites from RBL-2H3 cells upon Fc ϵ RI stimulation. RBL-2H3 cells were treated with Pam3CSK4 at the time of IgE sensitization and incubated for 2 h until stimulation with Ag. Culture supernatants were collected after 6 and 3 h of Ag stimulation to measure the concentrations of IL-13 and TNF- α , respectively. IL-13 production was sup-

Fig. 3. Effect of Pam3CSK4 on cytokine and LTC₄ production by RBL-2H3 cells. RBL-2H3 cells were sensitized with IgE for 2 h in the presence of 0–10 µg/ml (**a, b**) or 10 µg/ml (**c**) of Pam3CSK4 followed by stimulation with Ag. Concentrations of IL-13 (**a**), TNF-α (**b**) and LTC₄ (**c**) in the culture supernatants were determined by ELISA. Results are represented as the means ± SD of 2 (**a, b**) or 3 (**c**) independent experiments. *** *p* < 0.01, significantly different from the control without Pam3CSK4 treatment.

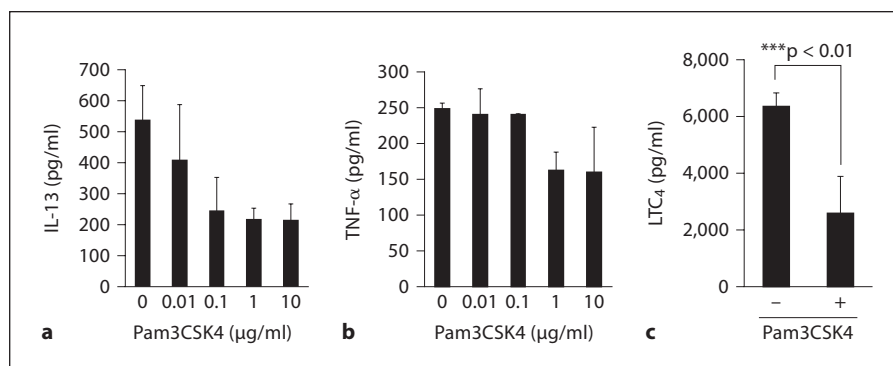
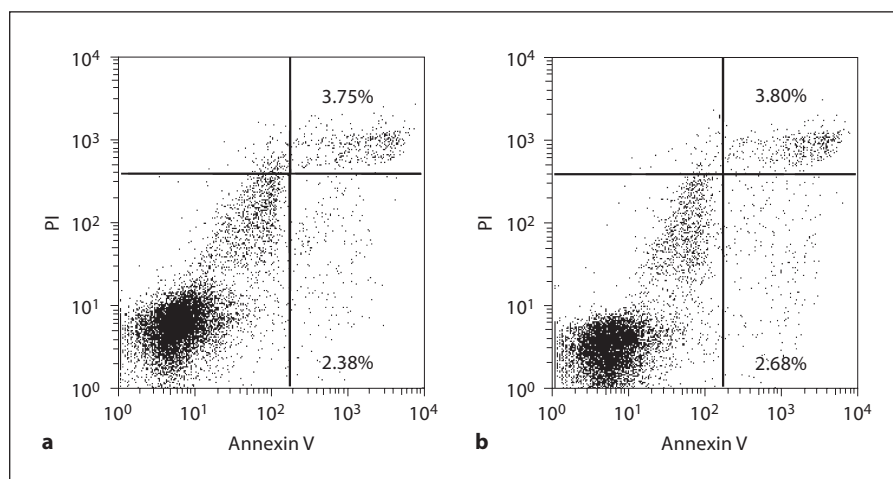


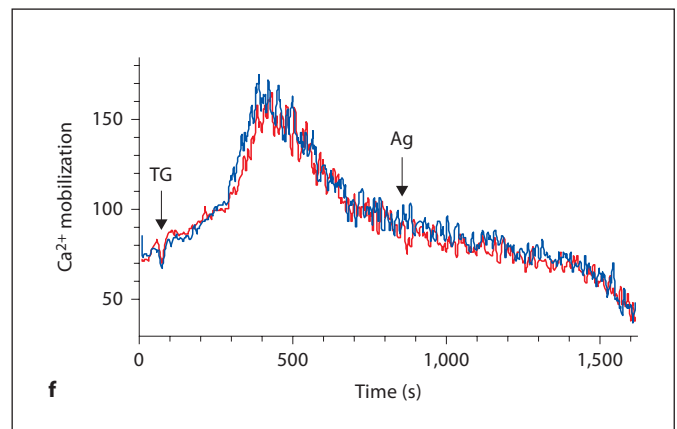
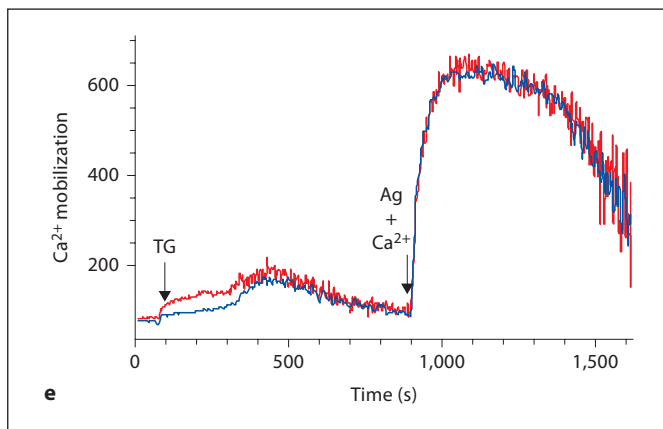
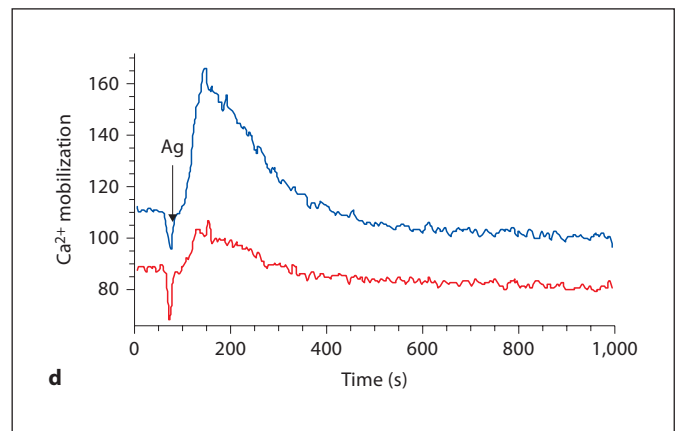
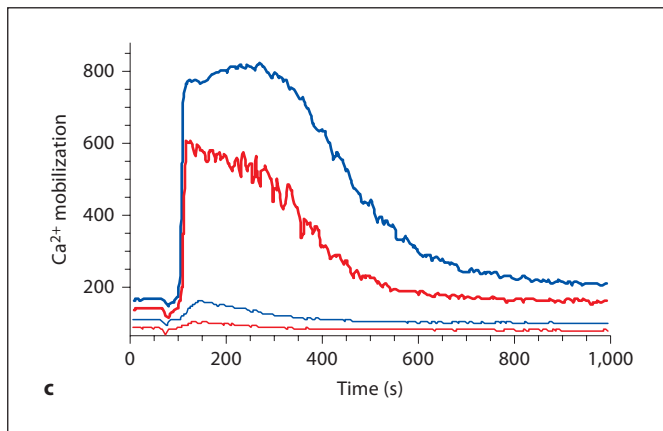
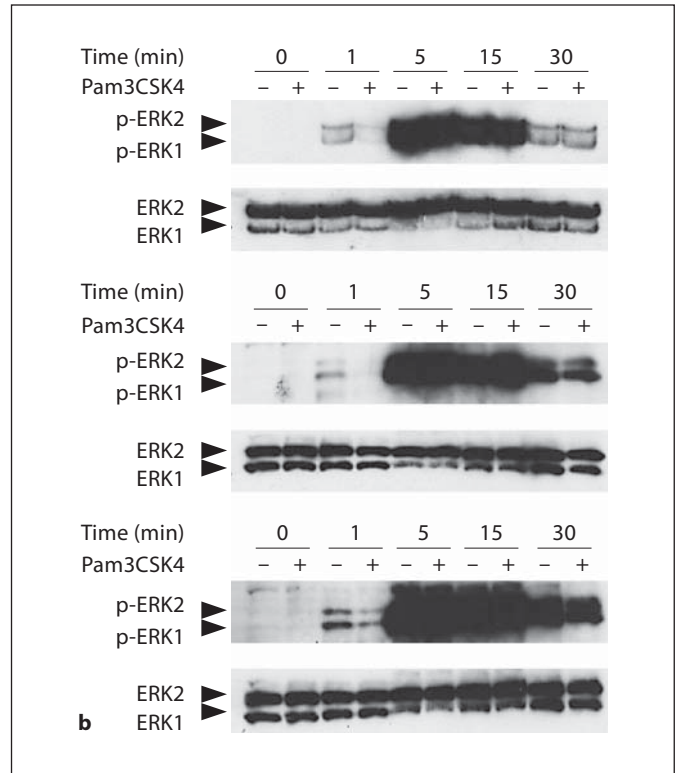
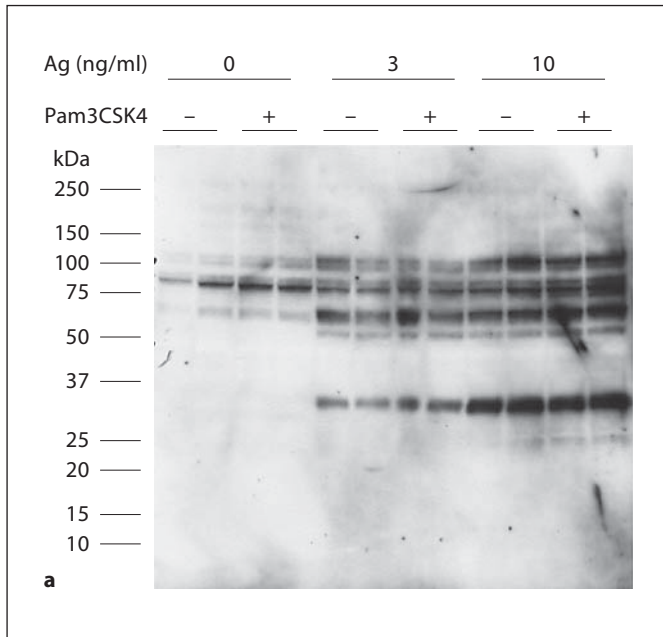
Fig. 4. Effect of Pam3CSK4 on cell viability. RBL-2H3 cells were treated with (**a**) or without (**b**) 10 µg/ml Pam3CSK4 for 2 h and stained with annexin V-FITC and propidium iodide (PI) for flow cytometric analyses. Results are representative of 2 independent experiments.



pressed by Pam3CSK4 in a dose-dependent manner (fig. 3a), while the production of TNF-α was only slightly suppressed by high doses of Pam3CSK4 (fig. 3b). Furthermore, secretion of LTC₄ into the culture supernatants of RBL-2H3 cells after 30 min of Ag stimulation was significantly inhibited by the addition of Pam3CSK4 (fig. 3c). Pam3CSK4 itself did not induce detectable levels of IL-13, TNF-α or LTC₄ production from RBL-2H3 cells at any concentrations used for the experiments without Ag stimulation (data not shown). The influence of Pam3CSK4 on cell viability was checked by double staining with annexin V-FITC and propidium iodide. As shown in figure 4, no facilitated induction of apoptosis or cell death was observed in the cells treated with Pam3CSK4 when compared with untreated cells, showing that the suppressive effects of Pam3CSK4 were not due to cytotoxic effects on RBL-2H3 cells. Collectively, these results indicated that the TLR2 ligand Pam3CSK4 suppressed the allergic reactions of mast cells in vitro.

To elucidate the underlying mechanisms for suppression of mast cell activation by Pam3CSK4, intracellular

signaling events including phosphorylation of signaling molecules and intracellular Ca²⁺ mobilization were analyzed. First, tyrosyl transphosphorylation which occurs in the early steps of signal transduction through FcεRI was analyzed 5 min after Ag stimulation by immunoblotting, employing anti-phosphotyrosine Ab. As shown in figure 5a, total tyrosine phosphorylation patterns were not significantly affected by Pam3CSK4 treatment, suggesting that Pam3CSK4 did not influence the activation of the early signaling molecules. On the other hand, phosphorylation of Erk, which belongs to the mitogen-activated protein kinase family and acts as one of the key signaling molecules mainly involved in the induction of proinflammatory cytokine production upon FcεRI engagement, was observed from 1 min after Ag stimulation, reached a maximum at 5 min and then decreased at 15 and 30 min. Phosphorylation of Erk1 and Erk2 was apparently lower in Pam3CSK4-treated cells than in untreated cells 1 min after Ag stimulation (fig. 5b). Calculation of band intensities of phosphorylated Erk1 and phosphorylated Erk2 normalized to those of Erk1 and Erk2,

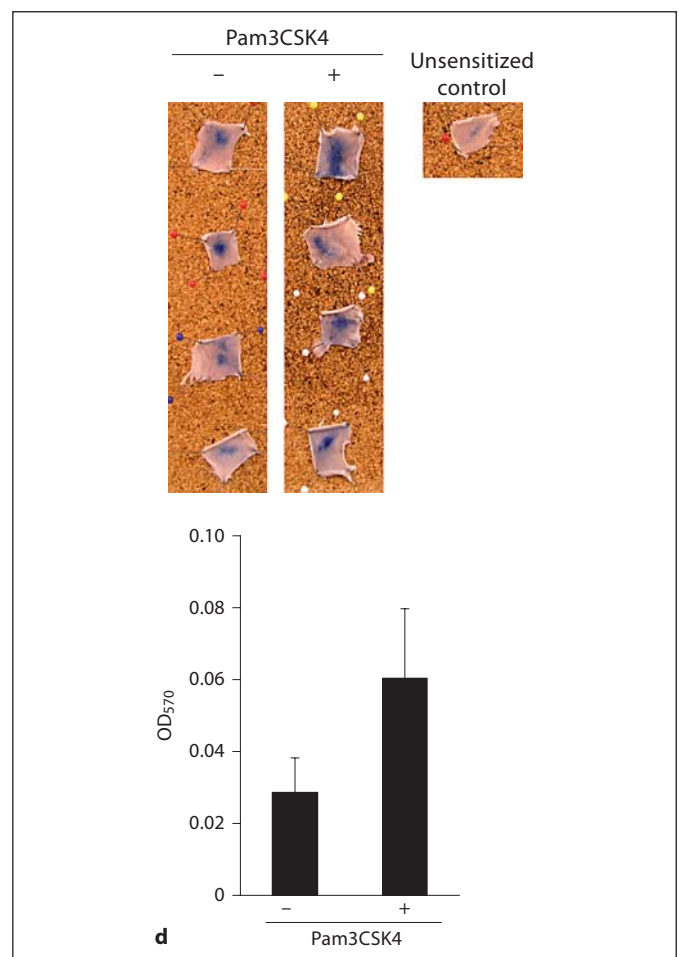
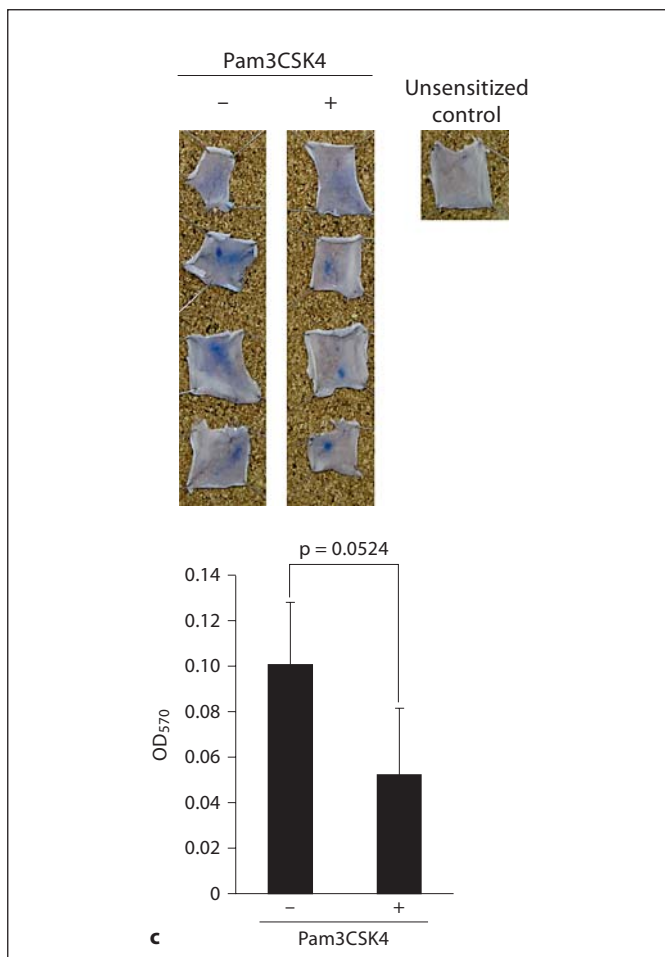
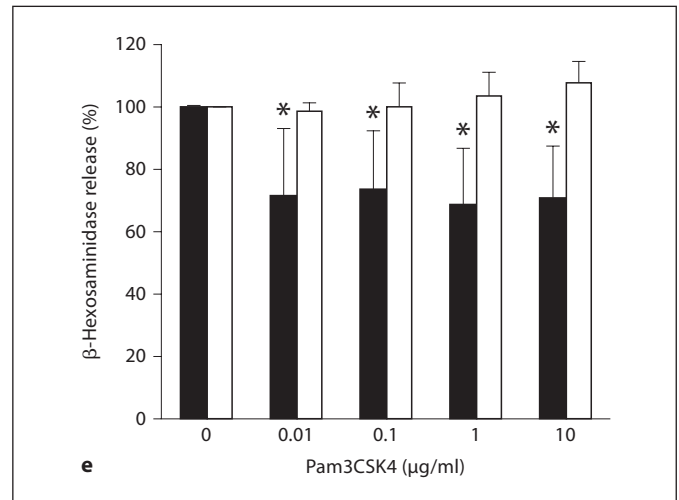
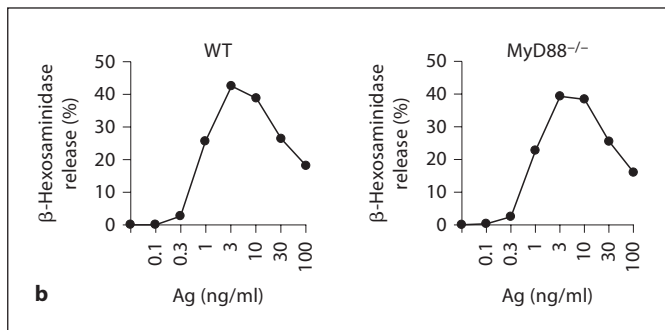
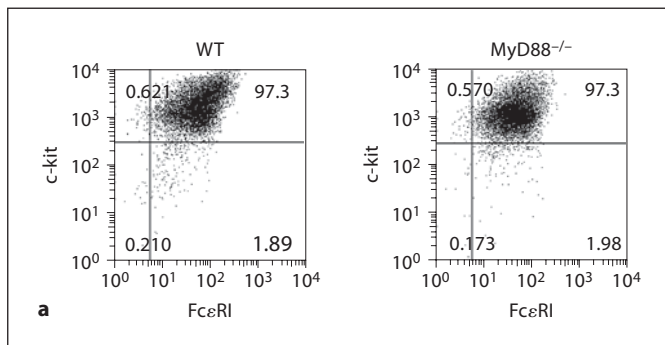


respectively, revealed that phosphorylation was significantly decreased by Pam3CSK4 treatment to $23.1 \pm 7.27\%$ for Erk1 and to $30.1 \pm 11.3\%$ for Erk2 1 min after Ag stimulation ($p < 0.0005$), but phosphorylation rates were not statistically different between Pam3CSK-treated and untreated cells 5, 15 and 30 min after Ag stimulation. These results indicated that activation of Erk was delayed in the cells treated with Pam3CSK4 compared with untreated cells. Furthermore, the intracellular Ca^{2+} concentration increased immediately upon Ag stimulation in both IgE-sensitized cells treated with and without Pam3CSK4, but the Ca^{2+} concentration remained elevated at a high level for a longer period in untreated cells than in the cells treated with Pam3CSK4 (see the data without EGTA expressed by bold lines in fig. 5c). Moreover, the effects of Pam3CSK4 on Ca^{2+} release from the ER and the subsequent influx of external Ca^{2+} activated by depletion of intracellular Ca^{2+} stores were analyzed separately. Ca^{2+} release from the ER upon Ag stimulation was measured in Ca^{2+} -free buffer supplemented with EGTA. As shown in figure 5d, Ca^{2+} release decreased in the cells treated with Pam3CSK4 compared with untreated cells. In contrast, the extracellular Ca^{2+} influx upon Ag stimulation after depletion of ER Ca^{2+} stores by thapsigargin was not affected by Pam3CSK4 treatment (fig. 5e,

f). Therefore, it was shown that Pam3CSK4 affected Ca^{2+} mobilization as a whole by suppressing Ca^{2+} release from the ER and, as a result, diminishing the subsequent influx of external Ca^{2+} . These findings indicated that decreased activation of Erk and insufficient retention of elevated intracellular Ca^{2+} could be involved in the suppression by TLR2 ligands of the allergic responses of mast cells.

Finally, the effects of Pam3CSK4 on a mast cell-induced increase in vascular permeability in vivo were investigated to examine whether the suppressive effects of Pam3CSK4 observed in vitro are able to affect the induction of inflammation in vivo. BMMCs from WT or MyD88^{-/-} C57BL/6 mice were prepared. The obtained BMMCs were mostly c-kit and FcεRI double positive (fig. 6a) and responded to IgE/Ag stimulation similarly (fig. 6b). BMMCs were sensitized with IgE in the presence or absence of Pam3CSK4 in vitro and transferred intradermally into the backs of W/W^v mice lacking mast cells. This was soon thereafter followed by the intravenous injection of Ag together with Evans blue to measure vascular permeability. The extravasation of Evans blue dye was normalized to that of the site where unsensitized BMMCs were transferred as control. The OD₅₇₀ value of this unsensitized control was almost equal to that of the site where PBS was injected alone (data not shown). When WT BMMCs were transferred, penetration of Evans blue dye was decreased to about half by the treatment with Pam3CSK4 ($p = 0.0524$), as shown in figure 6c. In contrast, when MyD88^{-/-} BMMCs were transferred, extravasation of Evans blue dye was not decreased by the treatment with Pam3CSK4 (fig. 6d). Interestingly, the dye extravasation tended rather to be increased by Pam3CSK4 treatment, although the results were not statistically significantly different ($p = 0.153$). Pam3CSK4 treatment alone did not induce the increase in vascular permeability, because extravasation of Evans blue into the site where unsensitized BMMCs treated solely with Pam3CSK4 were transferred was as small as that seen in the case of unsensitized control without Pam3CSK4 treatment (data not shown). The effects of Pam3CSK4 on in vitro degranulation of BMMCs from WT and MyD88^{-/-} mice were then compared (fig. 6e). The degranulation of WT BMMCs upon IgE/Ag stimulation was suppressed by Pam3CSK4, while that of MyD88^{-/-} mice was not affected by Pam3CSK4. These results indicated that the suppression of mast cell activation by Pam3CSK4 was sufficient to suppress the mast cell-mediated increase in vascular permeability in vivo, and that this effect was TLR2-dependent.

Fig. 5. Effect of Pam3CSK4 on intracellular signals. **a** RBL-2H3 cells were sensitized with IgE for 2 h in the presence or absence of Pam3CSK4. Five minutes after stimulation with Ag, cell lysates were prepared and immunoblotted with anti-phosphotyrosine Ab. Experiments were performed in duplicates for every condition throughout the steps from IgE sensitization to immunoblotting. **b** RBL-2H3 cells were sensitized with IgE for 2 h in the presence or absence of Pam3CSK4. Cell lysates were prepared at 0, 1, 5, 15 and 30 min after stimulation with Ag and immunoblotted with anti-phospho-ERK1/2 (**b**, top) and anti-ERK1/2 (**b**, bottom) Abs. Results of 3 independent experiments are shown. **c** RBL-2H3 cells were sensitized with IgE for 2 h in the presence (red lines) or absence (blue lines) of Pam3CSK4. Cells were loaded with Fluo-3AM in buffer containing Ca^{2+} (bold lines) or in Ca^{2+} -free buffer supplemented with EGTA (thin lines) and analyzed by flow cytometry. Ag was added to the cells at the time point indicated by an arrow. Data are representative of 3 independent experiments. **d** Part of figure **c** delineated by thin lines was magnified in a smaller range of the vertical axis to compare Ca^{2+} release from the ER between the cells with (red line) or without (blue line) Pam3CSK4 treatment. **e**, **f** After depleting the ER Ca^{2+} store by the addition of thapsigargin (TG) in Ca^{2+} -free buffer, Ag was added to the cells pretreated with (red lines) or without (blue lines) Pam3CSK4. Ag was added with (**e**) or without (**f**) Ca^{2+} . Data are representative of 4 independent experiments.



Discussion

We have shown that activation of mast cells upon FcεRI engagement can be suppressed by prestimulation of TLR2 on their surface, indicating a possibility that TLR2 ligands constituting probiotic bacteria exert antiallergic effects by directly suppressing the inflammatory responses of mast cells. Besides the well-known effects of probiotics on allergy, including the improvement in Th1/Th2 balance and the induction of regulatory T cells, our results place mast cells as another candidate target of probiotic microbes.

Strong stimulation through TLRs on mast cells causes the production of proinflammatory cytokines for the host defense reaction. However, it is thought that weak stimulation through TLRs on mast cells does not induce apparent cellular responses by itself but affects the responses of mast cells to other stimuli, as seen in our experiments. Actually, several reports have recently revealed that the functions of mast cells are modulated by stimulation through TLRs. For example, low doses of lipopolysaccharide enhanced mast cell-induced eosinophilic airway inflammation through TLR4 on mast cells,

indicating that lipopolysaccharide may become a risk factor for asthma [16]. Consistent with our results, Fehrenbach et al. [17] reported that prestimulation by Pam3CSK4 suppressed the degranulation of IgE-sensitized mast cells upon Ag stimulation, although Ca^{2+} mobilization (which was partially suppressed in our experiment) and tyrosine phosphorylation (which was not influenced in our study) were almost completely inhibited in their study. The difference is thought to be dependent on the strength of FcεRI engagement which leads to different intracellular signals, including negative feedback signals mediated by phosphatases upon strong FcεRI engagement, as well as the period of Pam3CSK4 treatment – 15 s in the study of Fehrenbach et al. [17] and 2 h in our study. On the other hand, Qiao et al. [18] reported that simultaneous stimulation of FcεRI and TLR4 or TLR2 synergistically augments the cytokine production but not the degranulation or release of arachidonic acid from IgE-primed mast cells. They added TLR ligands including Pam3CSK4 to the cells together with Ag. Collectively, the effects of stimulation through TLRs on the responses of mast cells upon IgE/Ag stimulation are different depending on the types of TLRs, the timing of TLR stimulation and the strength of the subsequent FcεRI engagement. Lasting exposure to TLR2 ligands prior to FcεRI stimulation, as it was found in our experimental conditions, can suppress the allergic reactions of mast cells upon Ag stimulation, and this may be the case for intestinal commensal bacteria or orally administered probiotics. Recently, Magerl et al. [19] reported that high doses of nonpathogenic commensal *Escherichia coli* also inhibited the degranulation of murine mast cells. In contrast, acute exposure to noncommensal or infectious microbes may synergistically increase some responses of mast cells upon FcεRI engagement. These findings may be associated in part with reports that allergic infants have an aberrant intestinal microbiota [20–24], although whether constituents of intestinal commensal bacteria reach distal sites outside the gut (i.e. in cases not involving intestinal allergy) is debatable.

In addition to the FcεRI-mediated reactions, various functions of mast cells are affected by stimulation through TLRs. Adhesion or cell surface expression of FcεRI, both of which can influence the allergic responses of mast cells, were reduced by stimulation via TLR3 and TLR2, respectively [25, 26]. Furthermore, microbial components may also influence the terminal differentiation of mast cells in the peripheral tissues through TLRs on their surface, because it is known that inflammatory signals are required for the maturation of mast cells [27]. For in-

Fig. 6. Effect of Pam3CSK4 on mast cell-induced increase in vascular permeability in vivo. **a** BMMCs were prepared from WT and MyD88^{-/-} mice. Cell surface expression of c-kit and FcεRI on BMMCs was analyzed by flow cytometry using PE-Cy7-labeled anti-c-kit Ab and Alexa 488-labeled IgE. **b** In vitro degranulation of IgE-sensitized BMMCs upon the stimulation with the indicated concentrations of Ag was determined by measuring the release of β-hexosaminidase. Values are expressed as percentages of the release to the total β-hexosaminidase content following subtraction of background values without Ag stimulation. **c, d** BMMCs prepared from WT (**c**) and MyD88^{-/-} (**d**) mice were sensitized with IgE in the presence or absence of 1 μg/ml Pam3CSK4 for 2 h and injected intradermally into the backs of W/W^v mice. After intravenous administration of Ag together with Evans blue dye, mice were sacrificed to evaluate the permeation of Evans blue into the skin through blood vessels. Evans blue permeated into each skin section was extracted by N,N-dimethylformamide and quantified colorimetrically. Absorbances at 570 nm were normalized by subtraction of those observed when unsensitized BMMCs were transferred. Data are represented as the means ± SD (n = 4 for each group). **e** The effects of Pam3CSK4 on in vitro degranulation of IgE-sensitized BMMCs from WT (black bars) and MyD88^{-/-} (white bars) mice upon stimulation with 3 ng/ml Ag were examined. Relative degranulation rates to those of the cells solely stimulated with IgE/Ag in the absence of Pam3CSK4 are expressed. Results are represented as the means ± SD of 3 or 4 independent experiments. * p < 0.05, significantly different from the control without Pam3CSK4 treatment.

stance, terminal differentiation or maturation of mast cells in the intestinal mucosa can be determined by intestinal commensal bacteria. It is suggested that probiotics possibly regulate allergic reactions in an indirect manner through these modulations in addition to the direct regulation.

The suppressive effect of Pam3CSK4 was shown to be mediated through TLR2 on the surface of mast cells, because the effect was MyD88 dependent (fig. 6). On the other hand, the suppression by Bp was partially dependent on MyD88 in an in vitro degranulation assay employing WT and MyD88^{-/-} BMMCs (data not shown). The *Bifidobacterium* components include additional ligands for other TLRs and other receptors such as NOD-like receptors, known as a family of intracellular sensors for microbial motifs [28]. It is thought that, besides TLR2, Bp also acts on these cell surface and intracellular receptors, because it has been observed that mast cells have an ability for the intracellular uptake of bacteria [29–31]. It has been reported that sonicated components of Bp stimulate the proliferation of mouse lymphoid cells and that its water-soluble, high molecular weight fraction has strong immunopotentiating activities [15]. We have also observed that oral administration of Bp decreased serum IgE levels in a murine model of allergy (unpublished data). It is expected that Bp has probiotic properties depending on these various characters in addition to the effect on mast cells.

The suppression of in vitro degranulation by Pam3CSK4 was dependent on its concentrations in RBL-2H3 cells (fig. 1c), but the dose dependency was not observed in BMMCs within the concentrations used for the assay (fig. 6e). The dose dependency may be observed at lower concentrations (<0.01 µg/ml) in BMMCs. Namely, the sensitivity of BMMCs to Pam3CSK4 may be higher than that of RBL-2H3 cells, although the maximal inhibition rate was not larger than that seen in RBL-2H3 cells.

IgE-sensitized mast cells immediately respond to Ag by releasing chemical mediators such as histamine stored in their granules, as well as by the generation of arachidonic acid metabolites including leukotrienes and prostaglandins. They also produce proinflammatory cytokines including TNF-α and IL-6 and Th2 cytokines including IL-4 and IL-13 through gene expression steps, which leads to the late-phase reaction. Since Pam3CSK4 suppressed degranulation, LTC₄ production and cytokine production (fig. 1, 3), but apparently did not affect the total tyrosine phosphorylation patterns (fig. 5a), it is thought that an upstream signaling event which is not just proximal to FcεRI is suppressed by TLR2 ligands.

Alternatively, multiple targets may be inhibited by them. Most of the suppressive effects are thought to be mediated by such inhibition of intracellular activation signals upon IgE/Ag stimulation. However, suppression of the degranulation reaction in cells treated with high doses of Pam3CSK4 may be mediated in part by diminishing the capacity of the cells to produce the mediators secondarily with previous subtle activation, because Pam3CSK4 used at the high concentration induced slight degranulation of cells although the total degranulation of Pam3CSK4-treated cells (degranulation caused by Ag plus that by Pam3CSK4) was still smaller than that of untreated cells. Although the suppressive effects observed in vitro were relatively mild, as the anti-allergic effects of probiotics usually are, they are thought to have significance because mast cell-induced inflammatory reactions in vivo could be alleviated (fig. 6). Further analyses will reveal the nature of mechanisms underlying the suppression of mast cell activation by TLR2 ligands.

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