



MyD88 but Not TLR2, 4 or 9 Is Essential for IL-12 Induction by Lactic Acid Bacteria

Shintaro ICHIKAWA,^{1,†} Rei FUJII,¹ Daisuke FUJIWARA,¹ Yutaka KOMIYAMA,¹
Tsuneyasu KAISHO,² Masahiro SAKAGUCHI,³ and Yutaka KONISHI¹

¹Central Laboratories for Frontier Technology, Kirin Holdings Company Ltd.,
1-13-5 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

²Laboratory for Host Defense, RIKEN Research Center for Allergy and Immunology,
1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

³Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University,
1-17-71 Fuchinobe, Sagami-hara, Kanagawa 229-8501, Japan

Received July 2, 2007; Accepted August 17, 2007; Online Publication, December 7, 2007
[doi:10.1271/bbb.70414]

Although lactic acid bacteria (LAB) affect the immune system, for example, having an anti-allergic effect, little is known about the actual mechanisms of immune modulation. Toll-like receptors (TLRs) recognize conserved microbial molecular patterns, and are presumed to be involved in the recognition of LAB. However, there are few detailed reports examining the relationships between TLR and LAB. We measured here production of IL-12, a cytokine considered to play an important role in anti-allergic effects, induced by *Lactobacillus paracasei* strain KW3110 and other typical LAB by cells from TLR2-, TLR4-, TLR9- and myeloid differentiation factor 88 (MyD88)-deficient mice. Unexpectedly, similar cytokine production from wild-type and TLR2-, 4- and 9-deficient mice was observed. In contrast, cells from MyD88-deficient mice failed to respond to stimulation with LAB. It is therefore concluded that although LAB, including strain KW3110, are not likely to be recognized by TLR2, 4 or 9, MyD88 is essential for the response to these bacteria.

Key words: lactic acid bacteria; toll-like receptor; interleukin-12

A number of studies has indicated that lactic acid bacteria (LAB) have anti-allergic effects in certain cases.¹⁻⁴⁾ We have previously reported that the KW3110 strain of *Lactobacillus paracasei* had the most potent IL-12-induction efficacy of more than 100 other LAB species tested. Furthermore, when orally administered to mice, this strain was found to inhibit IgE production *in*

vivo.⁵⁾ Moreover, orally administered KW3110 improved the allergic symptoms in an atopic dermatitis model (Wakabayashi *et al.*, manuscript submitted for publication). One of the key factors contributing to these anti-allergic effects is believed to be IL-12 produced by the antigen-presenting cells (APCs). This induces Th1 responses and reduces IL-4 and IgE production *in vitro* and *in vivo*.⁶⁻⁹⁾ Although KW3110 and other LAB are potent IL-12 inducers, the mechanism for this activity has not been identified.

Toll-like receptors (TLRs) comprise a family of pattern-recognition receptors that recognize specific molecular patterns related to microbial components.^{10,11)} TLR2 recognizes microbial lipoteichoic acid, the peptidoglycan of Gram-positive bacteria and lipoproteins, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, while TLR9 is the receptor for bacterial CpG-DNA.¹²⁾ These receptors act as the first line of defense against many bacteria and play an important role in the innate immune system. Interaction between TLRs and their ligands leads to the activation of several signaling cascades such as the NF- κ B pathway, followed by cytokine production including IL-12 and upregulation of the costimulatory molecules.¹³⁾ It is known that signal transduction by the members of this family (except for TLR3) requires the adapter molecule, myeloid differentiation factor 88 (MyD88),¹⁰⁾ and that MyD88-deficient animals fail to respond to pathogenic microbial infection.¹⁴⁾

As LAB, including strain KW3110, are Gram-positive bacteria, it was anticipated that they would be recognized by these TLRs. In fact, several studies have

[†] To whom correspondence should be addressed. Fax: +81-45-788-4047; E-mail: Shintaro.Ichikawa@kirin.co.jp

Abbreviations: APC, antigen-presenting cell; BM-DC, bone marrow-derived dendritic cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte/macrophage colony-stimulating factor; LAB, lactic acid bacteria; LPS, lipopolysaccharide; MLN, mesenteric lymph node; MyD88, myeloid differentiation factor-88; NOD, nucleotide-binding oligomerization domain; PE, phycoerythrin; PP, Peyer's patch; TLR, toll-like receptor; TNF, tumor necrosis factor; WT, wild-type

suggested that peptidoglycan or CpG-DNA derived from lactobacillus did indeed induce the activation of innate immunity.^{15,16} In these reports, however, the analyses were performed with purified microbial components, rather than whole organisms. It is therefore not yet clear how whole LAB are recognized by APCs. Here, using TLR-deficient mice, we examined how heat-killed LAB are recognized to induce IL-12 production. Unexpectedly, no loss of recognition or cytokine induction was apparent in TLR2-, 4- or 9-deficient mice stimulated by strain KW3110 or any other immunomodulating LAB. In contrast, MyD88-deficient mice did not respond to LAB at all. We also examined the compensation between each TLR-mediated IL-12 induction, and considered that it was not likely to occur in KW stimulation. It is therefore possible that MyD88-dependent but not TLR2-, 4- or 9-dependent signaling pathways are responsible for the recognition of both KW3110 and other LAB.

Materials and Methods

Mice. Male C57BL/6 and BALB/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Male MyD88-, TLR2-, TLR4- and TLR9-deficient mice with the C57BL/6 genetic background were obtained from Oriental Bioservice (Kyoto, Japan). The experiments were performed in accordance with the guidelines for care and use of laboratory animals of Kirin Holdings Co., Ltd. (Tokyo, Japan).

Materials. LPS derived from *Salmonella typhosa* was purchased from Sigma (St. Louis, MO, USA). Oligodeoxynucleotides containing unmethylated CpG motifs (1668) were obtained from Hycult Biotechnology (Netherlands). Synthetic lipoprotein FSL-1 was purchased from InvivoGen (San Diego, CA, USA). Fluorescein (FITC)-conjugated anti-mouse CD40 (clone HM40-3), CD80 (clone 16-10A1) and CD86 (clone GL1) were purchased from e-Bioscience (San Diego, CA, USA). phycoerythrin (PE)-conjugated anti-mouse CD11c (clone HL3) and FITC-conjugated anti-mouse CD11b (clone M1/70) were purchased from BD PharMingen (San Diego, CA, USA). Anti-mouse CD11c microbeads were purchased from Miltenyi Biotec (Germany). The heat-killed bacterial strains used in this study (*Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, *Lactobacillus acidophilus* L-92, *Lactobacillus johnsonii* Nestle and *Lactobacillus paracasei* KW3110) were prepared as described in a previous study.⁵

Preparation of bone marrow-derived DCs. To prepare bone marrow-derived dendritic cells (BM-DCs) *in vitro*, BM cells were removed from the femora and tibia and passed through a nylon mesh. Cells were then cultured for 8 days in 10-cm non-tissue culture-treated dishes with an RPMI 1640 medium (Sigma) supplemented with

5% fetal calf serum (FCS; Roche, Switzerland), penicillin/streptomycin (Sigma) and 20 ng/ml of the murine granulocyte/macrophage colony-stimulating factor (GM-CSF; Kirin Holdings, Japan). After 4 days of culture, an equal volume of 20 ng/ml of GM-CSF/5% FCS RPMI was added to the dish. At the end of the culture, the non-adhering cells were collected and incubated with anti-mouse CD11c microbeads. The CD11c+ cells were then separated by autoMACS (Miltenyi Biotec), those cells being used as BM-DCs with a purity >95%.

Cell culture and cytokine production. Cells from the spleen, mesenteric lymph nodes (MLN) or Peyer's Patch (PP) were suspended at 2.5×10^6 /ml in 10% FCS RPMI and cultured for 72 h with or without 1 µg/ml of the different bacterial strains. BM-DCs were suspended at 5×10^5 /ml in 5% FCS RPMI and cultured with 1 µM CpG, 1 or 10 µg/ml of LPS, 1 µg/ml of FSL-1, 1 µg/ml of KW3110 or 1 µg/ml of NRIC 1942 alone or in combination for 24 h. At the end of the culture, each supernatant was collected and BM-DCs were harvested for a FACS analysis. Cytokines were measured by using commercially available enzyme-linked immunosorbent assay kits for IL-12p70, IL-10 and TNF-α (BD PharMingen).

Results

Similar levels of cytokine production were induced by lactobacillus stimulation of the cells from TLR2-, 4-, or 9-deficient and wild-type mice

TLRs are considered as key players in the induction of cytokine production by microbial stimulation. TLR2 and 4 are thought to recognize components of the microbial cell wall, whereas TLR9 is considered to recognize CpG DNA.¹⁰ To evaluate the importance of TLR for inducing IL-12 production by KW, we examined the responses of cells from TLR2-, 4- and 9-deficient mice. Unexpectedly, the spleen and MLN cells from the TLR2-, 4- or 9-deficient mice secreted the same or even larger amounts of IL-12 than the wild-type (WT) mice (Fig. 1A). The production of IL-12 decreased to some extent in PP cells from the TLR2- and 9-deficient mice, compared to that of the WT mice, but the difference was not significant. Furthermore, in addition to IL-12, KW also induced similar amounts of tumor necrosis factor (TNF)-α and IL-10 in the TLR2-, 4- or 9-deficient mice to the WT mice (Fig. 1B). To investigate whether cytokine production by cells from the TLR-deficient mice was specific for KW3110, we next used typical immunomodulatory LAB as stimuli. No substantial differences in IL-12, TNF-α and IL-10 production by WT or TLR2-, 4-, or 9-deficient splenocytes, each stimulated by *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. johnsonii* or *L. paracasei*, were seen (Fig. 1C, not shown for TNF-α and IL-10). These data suggest that TLR2-, 4- or 9-deficiency was not crucial for either IL-

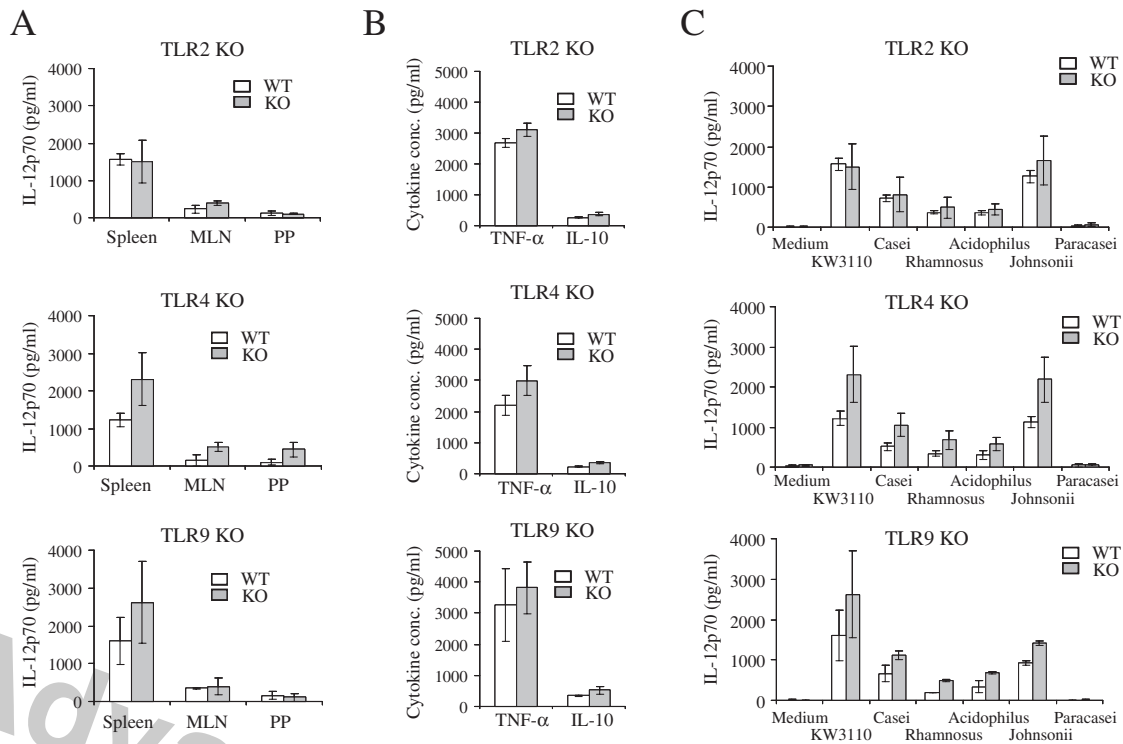


Fig. 1. Lactobacilli including *L. Paracasei* Strain KW3110 (KW) Generally Induced Cytokine Production by Spleen, MLN and PP Cells in a TLR2-, 4- or 9-Independent Manner.

A, Spleen, MLN or PP were removed from WT, TLR2-, 4- or 9-deficient mice and cultured with 1 μ g/ml of KW3110 for 72 h. The levels of IL-12p70 in the culture supernatants were determined by ELISA. B, The IL-10 and TNF- α concentrations in culture supernatants from WT, TLR2-, 4- or 9-deficient mouse splenocytes were determined by ELISA. C, Splenocytes from WT, TLR2-, 4- or 9-deficient mice were cultured with or without 1 μ g/ml of Lactobacilli (rhamnosus, casei, acidophilus, johnsonii or paracasei) for 72 h, and the levels of IL-12p70 in the culture supernatants were determined by ELISA. Open bars show WT mice and gray bars show TLR-deficient mice. Each data value is the mean $\pm$ SD of three mice.

12 production or other cytokine secretion following KW stimulation and, moreover, that this was likely to be a general finding for the immune responses against the other LAB species as well.

IL-12 production from BM-DCs upon KW stimulation was independent of TLR2, 4 or 9

DCs are potent IL-12-producing cells and likely to contribute to IL-12 production following KW stimulation. To analyze the correlation between KW stimulation and TLR recognition more fully, we examined the responses of BM-DCs from TLR-deficient mice. Immature BM-DCs induced from TLR2-, 4- or 9-deficient bone marrow cells were purified by MACS (>95%) and cultured with CpG, LPS (or FSL-1) or KW for 24 h. Although BM-DCs from the TLR2-, TLR4- and TLR9-deficient mice respectively showed impaired IL-12 production upon FSL-1, LPS and CpG stimulation, they still produced the same levels of IL-12 as the WT mice after KW stimulation (Fig. 2A). These results are therefore consistent with those just described for the splenocytes. To further investigate the maturation of BM-DCs, cells were harvested at the end of culture, and CD40 expression was analyzed by flow cytometry (Fig. 2B). Although the CD40 expression did not

increase with BM-DCs from TLR2-, 4- and 9-deficient mice after respective FSL-1, LPS, CpG stimulation, it did increase with BM-DCs from the TLR2- or 4-deficient mice with CpG stimulation and from TLR9-deficient mice with LPS stimulation. In contrast, increased CD40 expression was detected with BM-DCs derived from TLR-deficient mice of the same level as WT mice after KW stimulation. The expression of CD80 and CD86 was also increased with BM-DCs of TLR-deficient mice stimulated with KW (data not shown). These data are also consistent with the notion that TLR2, 4, or 9 was not necessary for the recognition of KW.

MyD88 was indispensable for IL-12 production after KW stimulation

Contrary to expectations, TLR deficiency did not affect IL-12 production upon KW stimulation. We therefore questioned whether TLR signaling was at all necessary for IL-12 production. Spleen, MLN, or PP cells from mice deficient in MyD88 (a known adapter molecule for TLR signaling) were cultured with KW, and IL-12 production assayed. As shown in Fig. 3A, IL-12p70 was undetectable in the spleen, MLN or PP cells from MyD88-deficient mice. The same result was

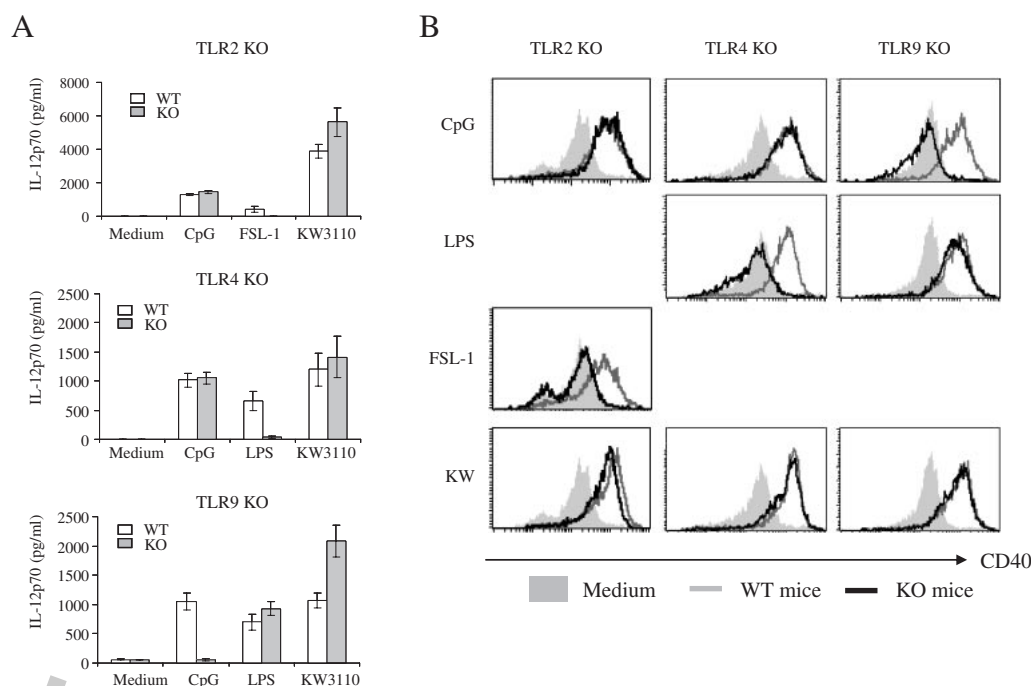


Fig. 2. Stimulation with Strain KW3110 Induced Normal Activation and Cytokine Production by BM-DCs Derived from TLR2-, 4- or 9-Deficient Mice.

Immature BM-DCs derived from TLR4- or 9-deficient mice were cultured with 1 μ M CpG, 10 μ g/ml of LPS or 1 μ g/ml of KW3110, and those from TLR2-deficient mice were cultured with 1 μ M CpG, 1 μ g/ml of FSL-1 or 1 μ g/ml of KW3110 for 24 h. A, The IL-12p70 concentrations of culture supernatants were determined by ELISA. Open bars show WT mice and gray bars show TLR-deficient mice. Each data value is the mean $\pm$ SD of three mice. B, Cultured BM-DCs were harvested and stained with the anti-CD40 mAb. CD40 up-regulation of BM-DCs was analyzed by flow cytometry. Gray shading represents non-stimulated BM-DCs, the gray line represents KW-stimulated BM-DCs of WT mice, and the black line represents KW-stimulated BM-DCs of TLR-deficient mice.

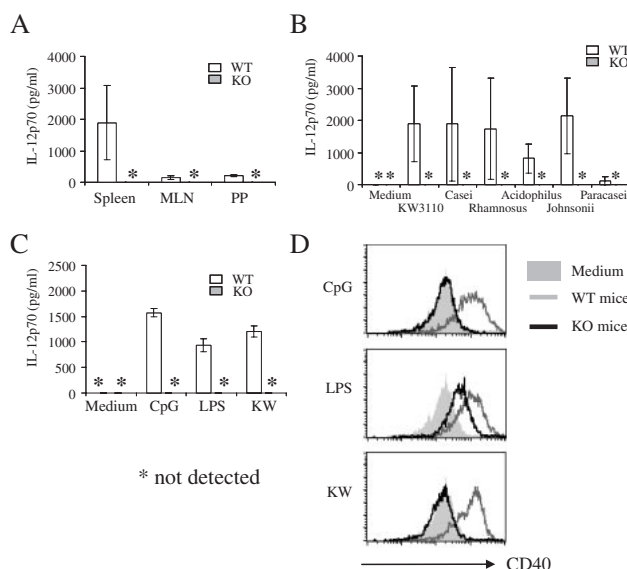


Fig. 3. MyD88 Was Necessary for IL-12 Induction and BM-DC Activation by the *L. Paracasei* KW3110 Strain.

Spleen, MLN or PP cells removed from WT and MyD88-deficient mice were cultured as described in Fig. 1. Figure 3A represents the IL-12p70 production of KW3110-stimulated spleen, MLN and PP cells. The IL-12p70 production by splenocytes stimulated with various strains of *Lactobacillus* was measured as described in Fig. 3B. Open bars show WT mice and gray bars show TLR-deficient mice. Each data value is the mean $\pm$ SD of three mice. BM-DCs from WT and MyD88-deficient mice were cultured, and the IL-12p70 production (C) and CD40 expression (D) were analyzed as described in Fig. 2. Gray shading represents non-stimulated BM-DCs, the gray line represents KW-stimulated BM-DCs of WT mice, and the black line represents KW-stimulated BM-DCs of TLR-deficient mice.

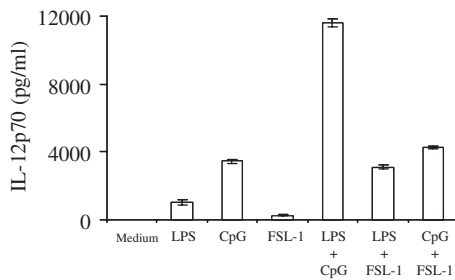


Fig. 4. IL-12 Production by BM-DCs Stimulated by Multiple Ligands.

Immature BM-DCs were derived from B57BL/6 mice and cultured for 24 h with LPS (1 µg/ml), CpG (1 µM) and FSL-1 (1 µg/ml) alone or in combination. The IL-12p70 concentration in each culture supernatants was measured. Each data value is the mean ± SD of triplicate cultures of one representative experiment of two.

obtained with splenocytes stimulated with other strains, and also with BM-DCs stimulated with KW (Fig. 3B and C). It is clear that MyD88 was essential for IL-12 induction after stimulation by KW and the other LAB strains. No increased CD40 expression with BM-DCs from MyD88-deficient mice was ever observed after KW stimulation, or after CpG stimulation (Fig. 3D). On the other hand, some up-regulation of CD40 was detected following LPS stimulation. This might suggest the presence of an MyD88-independent pathway of activation, as reported previously.¹⁴⁾

Combinations of TLR agonists synergistically induced IL-12 production from BM-DCs

To examine how different TLR ligands would work together for the induction of IL-12p70, BM-DCs derived from WT mice were cultured with LPS, CpG or FSL-1 alone or in combination, and IL-12 production was measured. A combination of LPS/CpG and LPS/FSL-1 showed synergy for IL-12 production (Fig. 4), while a combination of CpG/FSL-1 also showed at least an additive effect. These findings suggest that it was unlikely that signaling by the remaining TLRs responding to KW could compensate for deficient TLR signaling in the single TLR-knockouts.

Discussion

KW is a unique strain that induces strong IL-12 production and exerts anti-allergic effects by improving the Th1/Th2 balance.⁵⁾ In many studies, TLR2-, 4-, or 9-deficient mice have shown reduced IL-12 production following their stimulation with several kinds of microbial agents.^{17–20)} From those studies, it was expected that KW would also be recognized and induce IL-12 production via TLR. In the present study, however, IL-12 production from splenocytes of the TLR2-, 4- or 9-deficient mice was similar to that of WT mice following their KW stimulation (Fig. 1A). Unexpectedly, it was therefore demonstrated that TLR2-, 4- or 9-deficiency

had no effect on IL-12 production. We then prepared BM-DCs to clearly analyze the requirement for TLR on pure DCs. BM-DCs up-regulated such surface molecules as CD40, CD80 and CD86, and produced the same level of IL-12 in TLR2-, 4- or 9-deficient mice as in WT mice after their KW stimulation (Fig. 2). In addition, from the results for other strains with TLR-deficient splenocytes, these findings seem to apply to LAB in general (Fig. 1C). On the other hand, MyD88 was found to be clearly necessary for IL-12 production upon stimulation by KW as well as the other LAB (Fig. 3). Other than TLR, it has been reported that nucleotide-binding oligomerization domain (NOD) family members also sensed bacterial components.^{21,22)} However, it is also suggested that NOD family molecules are not likely to be involved in inducing IL-12 by KW and other LAB stimulation, because NOD have been assumed to be independent of the MyD88-related pathway.²³⁾

There are some previous reports concerning MyD88-dependent, TLR-independent immune responses following microbial stimulation.^{24–28)} Scanga *et al.* have reported that IL-12 production after stimulation with *Toxoplasma gondii* was similarly induced in TLR2- or TLR4-deficient mice as compared with WT mice.²⁶⁾ MyD88-dependent but TLR2-, 4- and 9-independent immune responses against *Mycobacterium tuberculosis* have also been reported.²⁴⁾ With respect to LAB in particular, Shida *et al.* have reported that *Lactobacillus casei* strain Shirota was recognized in a TLR2- or 4-independent and MyD88-dependent manner.²⁹⁾ However, these data were obtained only with peritoneal macrophages, and the involvement of TLR9 was not investigated. In the present study, we have shown that *Lactobacillus paracasei* strain KW3110 was recognized independently of TLR9, as well as TLR2 and 4. Recognition mechanisms are thought to be the same for the other LAB species, at least for splenocytes (Fig. 1C). In addition, we have also examined the responses of PP or MLN cells to KW3110 stimulation. These cells have important roles in immune responses against orally-administered antigens. Furthermore, the results with splenocytes are very similar to those with PP and MLN, supporting the conclusions of this study. This is the first report to show that LAB in general induced cytokine production in a TLR2-, 4- or 9-independent manner.

Since MyD88 was involved in the IL-1 signaling pathway,³⁰⁾ it might also be expected that KW3110 would induce IL-12 production via the IL-1 receptor. Indeed, Miller *et al.* have shown that *Staphylococcus aureus* induced immune activation in a TLR2-independent, MyD88- and IL-1R-dependent manner.²⁷⁾ However, our preliminary study using the IL-1R neutralizing antibody suggested that IL-1 had no influence on IL-12 production following KW3110 stimulation (not shown here).

One interpretation of the results of the present study is that it is conceivable that KW could be recognized by

each TLR receptor independently (2, 4 or 9) and that signaling *via* just one of these would be sufficient to trigger IL-12 production. Edelson *et al.* have reported that the immune responses against *Listeria monocytogenes* occurred normally in TLR2-deficient but not MyD88-deficient mice. They suggested that TLR4 or other TLRs might compensate for deficient TLR2 signaling.²⁵⁾ However, our studies showed that combinations of TLR2 and 4, TLR2 and 9, and TLR4 and 9 ligands had synergistic effects on IL-12 induction, consistent with the results of a previous study.³¹⁾ Even if KW3110 is assumed to induce IL-12 production by two or more TLRs, to compensate for the deficient TLR, some decrease in IL-12 production would be likely to occur when one TLR that is used for KW stimulation is missing. However, no such significant decrease in IL-12 production was observed in the TLR2-, 4- or 9-deficient mice. In addition, we have preliminary data showing that cells from TLR2/4 double knock-out mice produced normal levels of IL-12 after KW3110 stimulation. It thus seems unlikely that the deficient TLR signaling would be rescued by other TLRs that also recognize KW. Therefore, the similar IL-12 production from TLR deficient mice would not be due to this compensation. Further studies with TLR2/4/9 triple knock-out would be required to formally confirm this.

In conclusion, we examined the mechanisms for *in vitro* cytokine production following stimulation with KW3110 and other LAB. Our results demonstrate the absolute requirement of the MyD88 adaptor molecule for IL-12 production, but the lack of requirement of TLR2, 4 or 9. These responses were not specific only for the KW3110 strain, but more generally for other species of lactobacillus as well. The critical receptor for KW3110 is still unknown. Further studies on other MyD88-dependent receptors are required in future.

Acknowledgments

We thank S. Akira, M. Miyake and F. Yoshinaka for support during this work.

References

- 1) Xiao, J. Z., Kondo, S., Yanagisawa, N., Takahashi, N., Odamaki, T., Iwabuchi, N., Miyaji, K., Iwatsuki, K., Togashi, H., Enomoto, K., and Enomoto, T., Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial. *Clin. Exp. Allergy*, **36**, 1425–1435 (2006).
- 2) Shida, K., Takahashi, R., Iwadate, E., Takamizawa, K., Yasui, H., Sato, T., Habu, S., Hachimura, S., and Kaminogawa, S., *Lactobacillus casei* strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin. Exp. Allergy*, **32**, 563–570 (2002).
- 3) Matsuzaki, T., Yamazaki, R., Hashimoto, S., and Yokokura, T., The effect of oral feeding of *Lactobacillus casei* strain Shirota on immunoglobulin E production in mice. *J. Dairy Sci.*, **81**, 48–53 (1998).
- 4) Morita, H., He, F., Kawase, M., Kubota, A., Hiramatsu, M., Kurisaki, J., and Salminen, S., Preliminary human study for possible alteration of serum immunoglobulin E production in perennial allergic rhinitis with fermented milk prepared with *Lactobacillus gasseri* TMC0356. *Microbiol. Immunol.*, **50**, 701–706 (2006).
- 5) Fujiwara, D., Inoue, S., Wakabayashi, H., and Fujii, T., The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance. *Int. Arch. Allergy Immunol.*, **135**, 205–215 (2004).
- 6) Kips, J. C., Brusselle, G. G., Joos, G. F., Peleman, R. A., Devos, R. R., Tavernier, J. H., and Pauwels, R. A., Importance of interleukin-4 and interleukin-12 in allergen-induced airway changes in mice. *Int. Arch. Allergy Immunol.*, **107**, 115–118 (1995).
- 7) Sur, S., Lam, J., Bouchard, P., Sigounas, A., Holbert, D., and Metzger, W. J., Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *J. Immunol.*, **157**, 4173–4180 (1996).
- 8) Yssel, H., Abbal, C., Pene, J., and Bousquet, J., The role of IgE in asthma. *Clin. Exp. Allergy*, **28** (Suppl 5), 104–109; discussion 117–108 (1998).
- 9) Haas, H., Falcone, F. H., Holland, M. J., Schramm, G., Haisch, K., Gibbs, B. F., Bufe, A., and Schlaak, M., Early interleukin-4: its role in the switch towards a Th2 response and IgE-mediated allergy. *Int. Arch. Allergy Immunol.*, **119**, 86–94 (1999).
- 10) Takeda, K., Kaisho, T., and Akira, S., Toll-like receptors. *Annu. Rev. Immunol.*, **21**, 335–376 (2003).
- 11) Medzhitov, R., Toll-like receptors and innate immunity. *Nat. Rev. Immunol.*, **1**, 135–145 (2001).
- 12) Hemmi, H., Kaisho, T., Takeda, K., and Akira, S., The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J. Immunol.*, **170**, 3059–3064 (2003).
- 13) Akira, S., Takeda, K., and Kaisho, T., Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.*, **2**, 675–680 (2001).
- 14) Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S., Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.*, **166**, 5688–5694 (2001).
- 15) Sun, J., Shi, Y. H., Le, G. W., and Ma, X. Y., Distinct immune response induced by peptidoglycan derived from *Lactobacillus* sp. *World J. Gastroenterol.*, **11**, 6330–6337 (2005).
- 16) Takahashi, N., Kitazawa, H., Shimosato, T., Iwabuchi, N., Xiao, J. Z., Iwatsuki, K., Kokubo, S., and Saito, T., An immunostimulatory DNA sequence from a probiotic strain of *Bifidobacterium longum* inhibits IgE production *in vitro*. *FEMS Immunol. Med. Microbiol.*, **46**, 461–469 (2006).
- 17) Hou, L., Sasaki, H., and Stashenko, P., Toll-like receptor 4-deficient mice have reduced bone destruction following mixed anaerobic infection. *Infect. Immun.*, **68**, 4681–4687 (2000).
- 18) Feng, C. G., Scanga, C. A., Collazo-Custodio, C. M., Cheever, A. W., Hieny, S., Caspar, P., and Sher, A., Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune re-

- sponses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J. Immunol.*, **171**, 4758–4764 (2003).
- 19) Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A., and Sher, A., TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J. Exp. Med.*, **202**, 1715–1724 (2005).
 - 20) Akamine, M., Higa, F., Arakaki, N., Kawakami, K., Takeda, K., Akira, S., and Saito, A., Differential roles of Toll-like receptors 2 and 4 in *in vitro* responses of macrophages to *Legionella pneumophila*. *Infect. Immun.*, **73**, 352–361 (2005).
 - 21) Akira, S., Uematsu, S., and Takeuchi, O., Pathogen recognition and innate immunity. *Cell*, **124**, 783–801 (2006).
 - 22) Inohara, N., Chamaillard, M., McDonald, C., and Nunez, G., NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.*, **74**, 355–383 (2005).
 - 23) Park, J. H., Kim, Y. G., McDonald, C., Kanneganti, T. D., Hasegawa, M., Body-Malapel, M., Inohara, N., and Nunez, G., RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J. Immunol.*, **178**, 2380–2386 (2007).
 - 24) Su, S. B., Silver, P. B., Grajewski, R. S., Agarwal, R. K., Tang, J., Chan, C. C., and Caspi, R. R., Essential role of the MyD88 pathway, but nonessential roles of TLRs 2, 4, and 9, in the adjuvant effect promoting Th1-mediated autoimmunity. *J. Immunol.*, **175**, 6303–6310 (2005).
 - 25) Edelson, B. T., and Unanue, E. R., MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. *J. Immunol.*, **169**, 3869–3875 (2002).
 - 26) Scanga, C. A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E. Y., Medzhitov, R., and Sher, A., Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J. Immunol.*, **168**, 5997–6001 (2002).
 - 27) Miller, L. S., O'Connell, R. M., Gutierrez, M. A., Pietras, E. M., Shahangian, A., Gross, C. E., Thirumala, A., Cheung, A. L., Cheng, G., and Modlin, R. L., MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against *Staphylococcus aureus*. *Immunity*, **24**, 79–91 (2006).
 - 28) Harada, M., Magara-Koyanagi, K., Watarai, H., Nagata, Y., Ishii, Y., Kojo, S., Horiguchi, S., Okamoto, Y., Nakayama, T., Suzuki, N., Yeh, W. C., Akira, S., Kitamura, H., Ohara, O., Seino, K. I., and Taniguchi, M., IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. *J. Exp. Med.*, **203**, 2929–2937 (2006).
 - 29) Shida, K., Kiyoshima-Shibata, J., Nagaoka, M., Watanabe, K., and Nanno, M., Induction of interleukin-12 by lactobacillus strains having a rigid cell wall resistant to intracellular digestion. *J. Dairy Sci.*, **89**, 3306–3317 (2006).
 - 30) Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S., Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*, **9**, 143–150 (1998).
 - 31) Napolitani, G., Rinaldi, A., Bertonni, F., Sallusto, F., and Lanzavecchia, A., Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.*, **6**, 769–776 (2005).