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Review

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# Probiotics and immunology: separating the wheat from the chaff

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**Probiotics are live bacteria exhibiting health-promoting activities. Recent research has demonstrated that probiotics can prevent pathogen colonization of the gut and reduce the incidence or relieve the symptoms of various diseases caused by dysregulated immune responses. Probiotics seem to function by influencing both intestinal epithelial cells and immune cells of the gut, but the details of these effects are still being unraveled. Therefore, probiotics, through their effects on the host immune system, might ameliorate diseases triggered by disordered immune responses. Caveats remain and, because the beneficial effects of probiotics can vary between strains, the selection of the most suitable ones will be crucial for their use in the prevention or treatment of specific diseases.**

## Introduction

Humans are born in a sterile condition, but microorganisms from the mother and surrounding environment progressively begin to colonize the intestine of the infant after parturition. The gut microflora of the newborn are characterized by a predominance of bifidobacteria, and this is maintained during lactation. Weaning results in a remarkable alteration of gut microflora, characterized by the establishment of the adult type of gut microflora whose predominant bacteria are Firmicutes [1]. There is now evidence to suggest that dysregulation of the normal gut microflora composition or behavior might be related to certain disorders including colon cancer and inflammatory bowel diseases [2]. Therefore, foods having the ability to restore a normal gut microflora might be beneficial to the host. Probiotics are live bacteria with health-promoting activities, and several probiotics are used to make fermented foods such as cheese or yogurt. Indeed, there is accumulating evidence showing that probiotics can stabilize the ecosystem of intestinal microorganisms and modulate immune functions [3]. This article will focus on the beneficial effects of probiotics and try to present our current understanding of the mechanisms unpinning their immunomodulatory functions.

## Evidence for the beneficial effects of probiotics

Probiotics are live bacteria that can survive in the human intestine (see Box 1). The gut microflora are a complex ecosystem regulated by nutrients and physical conditions such as temperature, acidity and the gaseous composition of the intestinal lumen. The indigenous gut microflora is a 'cohabiting' partner to the host from birth to death. It fills a distinct ecological niche that can resist the colonization of exogenous pathogenic microorganisms. Moreover, normal immune system development can occur in response to stimuli from gut microflora. Therefore, individuals whose normal gut microflora are destabilized might in turn exhibit disrupted immune function and and/or become vulnerable to infectious diseases. Probiotics can assist in the recovery of gut microflora disturbed by a variety of causes and are expected to prevent or ameliorate certain diseases, at least in part, by modulation of the host immune system (see Table 1).

### Prevention of respiratory and enteric infection by probiotics

One of the beneficial effects exerted by probiotics is their defense against pathogenic infection. Because probiotics are usually resistant to gastric acid and bile salts, they can traverse the stomach and survive within the ileum [4]. They compete with pathogenic microbes for nutrients, and their metabolites (short chain fatty acids) can make the gut environment unsuitable for pathogens [5].

Several placebo-controlled, double-blind clinical trials have been performed to assess the anti-infectious abilities of probiotics (Table 2). Children and the elderly have often been the subject of studies to determine the clinical effects of probiotics because these groups tend to show the most dramatic responses to such treatments. For example, children attending a day care center who were supplemented with *Lactobacillus rhamnosus* GG showed a significantly reduced incidence of respiratory infection [6]. Similarly, feeding *L. reuteri* 55730 significantly reduced the incidence and duration of diarrhea in infants of 4–10 months old whose stool included rotavirus, *Shigella*, *Salmonella* or *Campylobacter* [7]. Similarly, short-term (5 d) administration with *L. rhamnosus* GG in children with acute diarrhea was found to shorten the duration of diarrhea

### Glossary

**AD:** atopic dermatitis
**CD:** Crohn's disease
**CLR:** C-type lectin receptor
**DC:** dendritic cell
**GALT:** gut-associated lymphoid tissue
**IEC:** intestinal epithelial cell
**IPAA:** ileal pouch-anal anastomosis
**NLR:** Nod-like receptor
**PBMC:** peripheral blood mononuclear cell
**TLR:** Toll-like receptor
**Treg:** regulatory T cell
**UC:** ulcerative colitis

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1471-4906/$ – see front matter  doi:10.1016/j.it.2008.07.011 Available online 4 October 2008

**Box 1. Prebiotics, probiotics and synbiotics**

Probiotics are defined as viable microorganisms, which, in sufficient numbers, alter the microflora of a host body compartment and thereby exert beneficial health effects [75]. For millennia, humans have selected probiotics to make fermentation products, and lactobacilli or bifidobacteria have often been used because these bacteria are both common and do not produce noxious substances in the human gut. Prebiotics on the other hand represent nondigestible food components capable of selectively stimulating the growth and/or activity of one or a limited number of bacteria, including probiotics, in the gastrointestinal tract and therefore exert a health-promoting effect [76]. Synbiotics are a combination of probiotics and prebiotics and are expected to exhibit more efficient health-promoting effects than those obtained by either of them alone. For instance, *Bifidobacterium lactis* HN019 or prebiotic (galactooligosaccharide) exhibited little effect on the composition of fecal microflora, but a synbiotic (combination of *B. lactis* HN019 and galactooligosaccharide) efficiently increased lactobacilli and bifidobacteria in feces [77]. Synbiotics containing *B. breve, Lactobacillus casei* Shirota and galactooligosaccharide increased lactobacilli and bifidobacteria in feces and improved the rate of body weight gain in patients with short bowel syndrome [78]. The accumulation of evidence-based information about synbiotics will enable us to determine the best synbiotics for each subject and disease setting.

[8]. Other independent studies have also demonstrated similar beneficial effects in the control or prevention of diarrhea with other probiotic strains [9,10]. Collectively, these results suggest that enteropathogenic infection in children can be ameliorated by continuous supplementation with probiotics.

The elderly are more susceptible to infection compared with young adults because of an age-related decline of immune function. With this in mind, the effects of probiotics on respiratory tract infection in the elderly have been evaluated (Table 2). A pilot study in the elderly showed that the duration of winter infections was shorter in subjects given fermented milk containing *L. casei* DN-114 001 than those of controls [11]. Another study demonstrated that dietary supplementation with *L. johnsonii* La1 reduced the duration of respiratory infection [12]. Similarly, even when healthy young adults were fed different probiotics (either a mixture of *L. gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3 and *B. bifidum* MF 20/5 [13] or *L. fermentum* CETC5716 [14]), the duration and severity of respiratory tract infections were also reduced. The detailed mechanisms of how supplementation with probiotics can reduce the incidence of respiratory tract infection remain to be clarified, but it has been suggested that this might be because of an increase of cytotoxic T cells or natural killer (NK) cells and augmentation of antibody levels after influenza vaccination [13,14].

Studies to assess the beneficial efficacy of probiotics have been performed in cancer patients or critically ill patients, because these groups can be particularly vulnerable to opportunistic infections. When patients who underwent surgical resection of colon or cervical cancers were supplemented with VSL#3 (a mixture including four strains of lactobacilli, three strains of bifidobacteria and one strain of streptococcus) or placebo throughout radiation therapy, the incidence of diarrhea was lower in the probiotics group [15]. In another clinical trial where patients admitted to an intensive care unit were fed VSL#3 or placebo, supplementation with probiotics tended to reduce the incidence of diarrhea and recovered serum IgG and IgA levels [16]. Taken together, much evidence supports the contention that probiotics impact the host, potentially via modulation of the immune system and/or competition with pathogens, and this helps reduce the incidence of enteric infection.

## Treatment of inflammatory bowel diseases with probiotics

Ulcerative colitis (UC) and Crohn's disease (CD) are idiopathic inflammatory bowel diseases (IBDs) (also see the article by Danese in this issue). UC is a relapsing, nontransmural inflammatory disease restricted to the colon, and CD is a relapsing, transmural inflammatory disease affecting the entire gastrointestinal tract. Healthy subjects are tolerant to their gut microflora. However, in IBD patients, this immunological tolerance to gut microflora is broken, and hypersensitivity to gut antigenic loads is crucial to pathogenesis [17]. The normal first line of treatment for patients with mild or moderate UC is the anti-inflammatory drug 5-aminosalicylic acid (mesalazine) [18], but several clinical studies to evaluate the therapeutic efficacy of probiotics in UC patients have been performed (Table 2). When patients with active UC were divided into two groups and given mesalazine or nonpathogenic *Escherichia coli* Nissle 1917, both treatments prevented the

**Table 1. Effect of probiotics on indigenous gut microflora and immune function in humans**

| Probiotics | Subjects | Number of patients | Duration | Outcome | Refs |
|---|---|---|---|---|---|
| **Stabilization of gut microflora** | | | | | |
| *Lactobacillus casei* Shirota | Healthy adults (40~65 years) | 10/group | 4 weeks | Increase of bifidobacteria | [80] |
| *L. rhamnosus* GG | Preterm neonates (<1500 g) | 15~24/group | 3 weeks | Increase of Gram-positive bacteria and anaerobic bacteria | [81] |
| *L. johnsonii* La1 | Healthy adults (20~22 years) | 11/group | 3 weeks | Increase of bifidobacteria and decrease of clostridia | [82] |
| *Bifidobacterium lactis* HN019 | Free-living elderly (60~87 years) | 14~19/group | 4 weeks | Increase of bifidobacteria and decrease of enterobacteria | [83] |
| **Modulation of immune system** | | | | | |
| *L. johnsonii* La1 | Healthy adults (21~57 years) | 14/group | 3 weeks | Increase of phagocytic activity in PBMCs[a] | [84] |
| *B. lactis* HN019 | Healthy elderly (63~84 years) | 15/group | 3 weeks | Increase of phagocytic activity and natural killer activity in PBMCs | [56] |
| *L. rhamnosus* GG | Healthy infants (<2 months) | 40/group | Until 12 months | Increase of cow's milk specific IgA-producing cells in PBMCs | [85] |
| *L. casei* Shirota | Healthy elderly (69~97 years) | 10/group | 3 weeks | Increase of natural killer activity in PBMCs | [57] |

[a]PBMC, peripheral blood mononuclear cell.

**Table 2. Key clinical effects of probiotics examined in human studies**

| Probiotics | Subjects | Number of patients | Duration | Outcome | Refs |
|---|---|---|---|---|---|
| **Prophylaxis or treatment of respiratory and gastrointestinal infection** | | | | | |
| *Lactobacillus rhamnosus* GG | Healthy children (1~6 years) | 282~289/group | 7 months | Decrease of incidence of respiratory infections | [6] |
| *L. reuteri* ATCC55730 | Healthy children (4~10 months) | 58~71/group | 12 weeks | Decrease of incidence and duration of diarrhea | [7] |
| *L. rhamnosus* GG | Children with diarrhea (3~36 months) | 91~100/group | 5 days | Decrease of duration of diarrhea | [8] |
| *L. casei* DN-114 001 | Free-living elderly (>60 years) | 180/group | 3 weeks | Reduction of duration of all pathologies due to infection | [11] |
| *L. johnsonii* La1 | Hospitalized elderly (>70 years) | 12/group | 12 weeks | Decrease of duration of respiratory infections | [12] |
| **Treatment of inflammatory bowel diseases** | | | | | |
| *Escherichia coli* Nissle 1917 | Patients with active UC[a] (18~80 years) | 57~59/group | 12 months | Prevention of relapse with efficacy equivalent to mesalazine | [19] |
| *L. rhamnosus* GG | UC patients in remission (mean 33 years) | 60~65/group | 12 months | Prevention of relapse more effectively than mesalazine | [20] |
| *Bifidobacterium breve* + *B. bifidum* | UC patients in remission (39~60 years) | 10~11/group | 12 months | Prevention of exacerbation in clinical symptoms | [21] |
| VSL#3 | UC patients after IPAA (18~65 years) | 20/group | 9 months | Prevention of relapse of pouchitis | [22] |
| **Prophylaxis or treatment of allergic symptoms** | | | | | |
| *L. rhamnosus* GG | Newborns with familial history of allergy | 77~82/group | 6 months | Decrease of incidence of AD at 2 year | [33] |
| *L. fermentum* VRI-033 PCC | Children with AD (6~18 months) | 28/group | 8 weeks | Alleviation of clinical severity | [34] |
| *B. longum* BB536 | Patients with pollinosis (mean 37 years) | 20/group | 14 weeks | Alleviation of allergic symptoms | [37] |
| *L. casei* Shirota | Patients with pollinosis (mean 39 years) | 60/group | 8 weeks | No significant effect (trend to delay the clinical symptoms) | [38] |
| *L. casei* DN-114 001 | Children with allergy (2~5 years) | 98/group | 12 months | Decrease of incidence of rhinitis | [39] |

[a]UC, ulcerative colitis; IPAA, ileal pouch-anal anastomosis; AD, atopic dermatitis.

relapse of disease with equivalent efficacy [19]. *L. rhamnosus* GG also exhibited a beneficial effect equivalent to mesalazine in terms of remission of UC patients [20]. Moreover, daily ingestion of bifidobacteria-fermented milk in conjunction with conventional treatment for 1 year reduced the cumulative exacerbation of UC compared with conventional treatment alone [21].

It is known that UC patients who undergo ileostomy closure after ileal pouch-anal anastomosis often suffer from inflammation around the pouch (pouchitis). Placebo-controlled clinical trials demonstrated that probiotics (VSL#3) could prevent the relapse of pouchitis in UC patients [22]. However, it is important to emphasize that not all the data in this area are consistent with clinical improvement; for instance, the administration of *L. johnsonii* La1 after ileo-cecal resection failed to prevent early recurrence of CD [23].

How probiotics can ameliorate the pathogenesis of UC remains to be elucidated. One possibility is that probiotics could decrease the level of bacterial species involved in the pathogenesis of UC. *Bacteroides vulgatus* has been found to increase in the colonic mucosa of UC patients and might contribute to the pathology of this disease [24]. Dietary supplementation with bifidobacteria-fermented milk reduced the ratio of *B. vulgatus* among the total Bacteroidaceae in feces, probably by normalizing gut microflora [21]. Therapy with VSL#3 was found to increase the total number of intestinal bacteria and restore the diversity of gut microflora, therefore stopping a preponderance of any one particular species of bacteria [25]. Aside from the possibility of normalizing the gut microflora, immunomodulation by probiotics might also be involved in the improvement of disease symptoms in IBD patients. It has been reported that consumption of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 supplemented yogurt for 30 d increased the proportion of $CD4^+CD25^+$ cells (regulatory T cells; Treg cells) and decreased the ratio of tumor necrosis factor α ($TNF\text{-}\alpha^+$) and interleukin 12 ($IL\text{-}12^+$) monocytes and dendritic cells (DCs) in the peripheral blood of IBD patients [26], although it is uncertain whether these changes in immunological parameters are connected with the clinical improvement. Further studies in this direction need to be carried out.

## Alleviation of allergic symptoms by probiotics

The 'hygiene hypothesis' postulates that the recent drastic increase of allergic diseases in industrialized countries is caused by decreased levels of childhood infection. Such infections would otherwise 'fine-tune' the immune system into an allergy-protective response. A related extrapolation of the hygiene hypothesis has speculated that changes in the gut microflora (e.g. caused by antibiotic use or components of the modern diet) could also be associated with the increase of allergic diseases. In support of this notion, murine studies have shown that commensal intestinal bacteria play a pivotal role in the normal development of immunological tolerance to orally administered antigens [27]. Furthermore, one case-controlled study has indicated that children with atopic dermatitis (AD) were less often colonized with lactobacilli and bifidobacteria compared with healthy children [28]. A subsequent prospective study showed that prevalence of bifidobacteria was less frequent in babies who developed allergic symptoms during the first year of life [29], suggesting that dysregulation of gut microflora might precede the appearance of allergic symptoms. Moreover, a cohort study showed that the presence of *Clostridium difficile* in the gut was associated with a higher risk of AD

[30]. A comparative analysis in another cohort study demonstrated that diversity in fecal microflora in infants with atopic eczema was reduced during the first 18 months of life [31], although development of atopic eczema was not associated with earlier or delayed colonization by any particular bacterial group [32]. Taken together, the data suggest that abnormalities in the gut microflora such as a decrease or increase of a particular bacteria species might be associated with some forms of allergy.

The management of allergic diseases by means of probiotics and their effects on gut microflora has been the subject of clinical interest (Table 2). The first large-scale placebo-controlled study demonstrating that supplementation of *L. rhamnosus* GG in newborn infants reduced the incidence of AD at 2 years of age [33] encouraged several similar clinical trials. Supplementation of diets with *L. fermentum* VRI-033 PCC reduced the severity of AD during childhood [34]. These results support the notion that specific probiotic strains are effective in prevention and treatment of certain forms of AD in infants. However, it should be noted that, in one large-scale prospective study using *L. acidophilus* LAVRI-A1, there was no reduction in the risk of AD and in fact even an increased risk of allergic sensitization in high-risk children [35].

The effects of probiotics on allergic rhinitis are controversial. Although a study in Europe proposed that supplementation of *L. rhamnosus* GG had no beneficial effects on birch-pollen allergy [36], a clinical trial in Japan revealed that supplementation of *B. longum* BB536 improved the symptoms of Japanese cedar-pollen allergy [37]. Another clinical study in Japan showed that a drink of *L. casei* Shirota-fermented milk slightly delayed the occurrence of allergy in the subgroup of patients with moderate-to-severe nasal symptoms, although the severity of pollinosis was not improved by probiotics [38]. In addition, supplementation with *L. casei* DN-114 001 exhibited a prophylactic effect on the incidence of allergic rhinitis in children [39]. Therefore, there is evidence suggesting that probiotics have the potential to alleviate the symptoms of allergic rhinitis, but not all patients seem responsive, probably caused by a wide range of genetic and environmental factors.

In general, the finding that probiotic supplementation to newborns is more effective in management of AD might be explained as follows: (i) the gut microflora of newborns can be more easily manipulated by probiotic supplementation compared with that of adults; (ii) development of the immune system in newborns is affected by intestinal microflora and therefore can be modified by probiotics and (iii) permeability of intestinal epithelia is damaged in children suffering from AD and probiotics are expected to improve the intestinal permeability with a resulting blockade of allergen uptake. Newborns might therefore be at a crucial stage when the gut microfloral community is initially constructed.

## Mechanisms of immunomodulation by probiotics

As mentioned above, probiotics serve to not only stabilize the gut microflora but can potentially modulate the function of immune cells. Microorganisms in the gut lumen are recognized and processed by the immune system through several routes. (i) They can attach to intestinal epithelial cells (IECs) and modulate their function directly. (ii) M (microfold) cells localized in the follicle-associated epi-

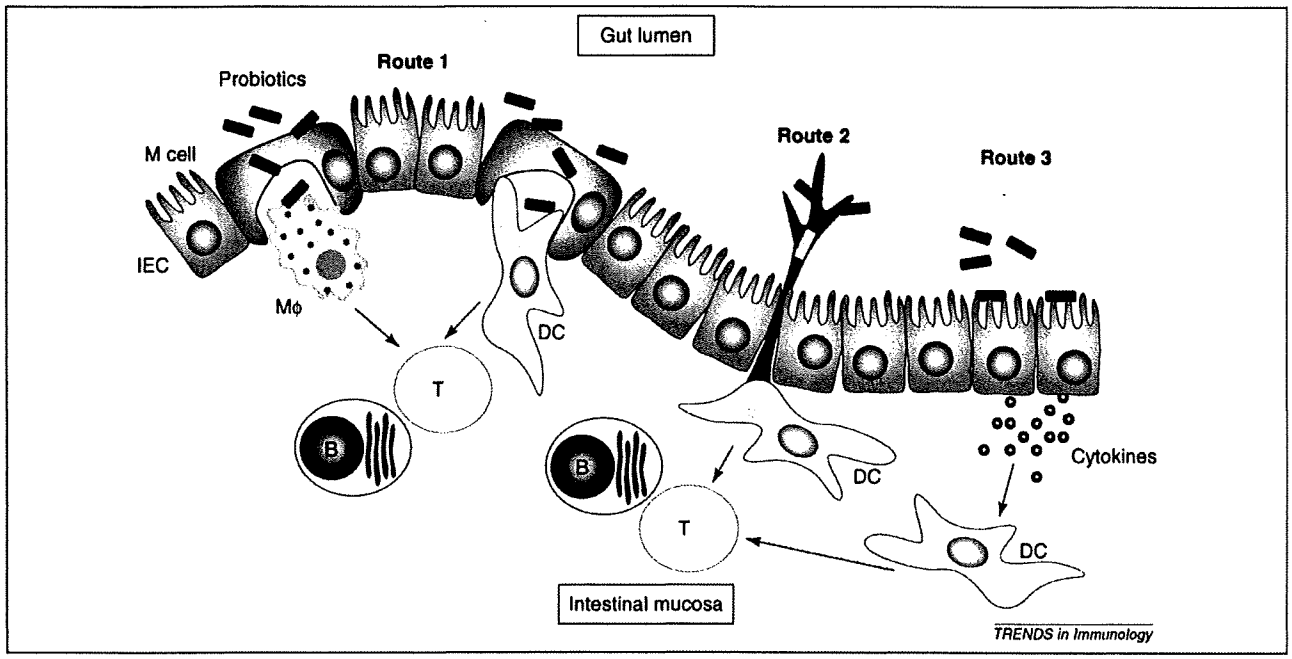


**Figure 1.** Three hypothetical pathways by which probiotics can trigger and modulate immune function in the intestine. **(a)** Specialized epithelial cells called M (microfold) cells in the follicle-associated epithelium covering Peyer's patches or in the villi can take up probiotics directly by transcytosis. Macrophages (Mϕs) or dendritic cells (DCs) are present immediately below M cells and then engulf probiotics and trigger immune responses. **(b)** DCs in the intestinal lamina propria have been found to extend their dendrites between intestinal epithelial cells (IECs) and might directly sample and process probiotics in the gut lumen. **(c)** Probiotics directly affect IECs to secrete an array of cytokines, which in turn modulate the immune functions of DCs, T cells and B cells in the gut-associated lymphoid tissue (GALT).

thelium overlying Peyer's patches can transport them to the immune cells in the subepithelial dome region immediately underneath. (iii) DCs in the lamina propria (LP) actively extend dendrites to sample microbes in the gut lumen. Although there is still far from complete understanding, probiotics might exert their immune-modulating functions through a similar set of pathways (Figure 1).

### Effect of probiotics on IECs

Under normal conditions, commensal bacteria inhabit the gut lumen and actively modulate the function of IECs without inducing inflammatory responses [40] (also see the article by Canny and colleagues in this issue). The cellular and molecular mechanisms underlying this normally clement coexistence between commensal and host are gradually being elucidated. Infection of the human epithelial cell line, Caco-2, by *Shigella flexneri* normally induces a proinflammatory response including chemokine production, but preincubation of Caco-2 cells with probiotic *L. casei* DN-114 001 downregulates the response induced by *S. flexneri* through the blockade of NF-κB activation [41]. TNF-α induces apoptosis of mouse colonic epithelial cells, but this can be suppressed by *L. rhamnosus* GG by activating the antiapoptotic protein Akt [42]. Furthermore, it has been reported that the probiotic mixture VSL#3 enhanced mucin secretion by the human colonic epithelial cell line, LS 174T, and in the rat colonic loop, which would probably have the effect of remodeling the barrier function of IECs [43]. Collectively, these results demonstrate that some probiotic strains can act directly on IECs to maintain the integrity of the epithelial barrier.

### Recognition of probiotics by immune cells

Phagocytic cells express pattern-recognition receptors such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and Nod-like receptors (NLRs) that are specific for various microbial components. After probiotics are incorporated into the lamina propria (LP) and Peyer's patches, the pattern recognition receptors expressed by macrophages and DCs of the GALT (gut-associated lymphoid tissue) can recognize them, resulting in cytokine secretion and expression of co-stimulatory molecules. *In vitro*, *B. bifidum* W32 and *B. infantis* W52 were shown to stimulate TLR2-expressing CHO (Chinese hamster ovary) cells to activate NF-κB [44], and lipoteichoic acid derived from *L. casei* Shirota has been shown to stimulate macrophages to secrete TNF-α by TLR2-mediated signaling [45]. Orally ingested *L. rhamnosus* Lr23 could promote the development of regulatory DCs in a TLR2-dependent manner [46]. TLRs other than TLR2 are also involved in the recognition of probiotics, because administration of VSL#3 could ameliorate the experimental induction of colitis in TLR2- and TLR4-deficient mice and wild-type mice but not in MyD88- and TLR9-deficient mice [47]. Some *Lactobacillus* strains were found to promote the differentiation of immature DCs into regulatory DCs *in vitro*, and this was dependent on a member of the CLR family [48]. Furthermore, a recent report showed that some other *Lactobacillus* strains were recognized through the Nod2 receptor [49]. Thus, probiotics express a variety of ligands recognized by TLRs, CLRs and NLRs of the host.

### Regulation of cytokine production by probiotics

Macrophages and DCs exposed to probiotics can be observed to secrete a variety of cytokines. Chief among these are IL-12 and IL-10, which are key to controlling the balance of the immune response, because the former augments cellular immunity, whereas the latter suppresses inflammatory responses. Comparative analyses have revealed that the abilities of *Lactobacillus* strains to induce IL-12 production by macrophages are highly variable [50,51]. For instance, an oligodeoxynucleotide (ODN) containing a unique TTTCGTTT motif purified from *L. rhamnosus* GG was found to strongly induce IL-12 production in murine splenocytes and suppress OVA-specific IgE response in mice [52,53]. By contrast, the insoluble intact cell wall, but not the soluble one, of *L. casei* Shirota activated murine peritoneal macrophages to produce higher amounts of IL-12, suggesting that macrophages need recognition of the three-dimensional structure of certain *Lactobacillus* strains for abundant production of IL-12 [51]. *Lactobacillus* strains belonging to the *L. casei* group such as *L. casei* Shirota, *L. rhamnosus* GG and *L. paracasei* KW3110 have been shown to induce IL-12 production by TLR2-, TLR4- and TLR9-deficient macrophages but not MyD88-deficient macrophages [51,54]. Recently, it has been reported that *Streptococcus pyogenes*, a pathogenic Gram-positive bacterium, could induce proinflammatory cytokines in TLR2-, 4- and 9 triple-deficient and wild-type macrophages but not MyD88-deficient macrophages [55]. These results suggest a novel idea that some probiotic strains as well as *S. pyogenes* might be recognized by an as yet unknown MyD88-dependent receptor and thereby trigger IL-12 production. Further studies are required to dissect the receptors responsible for transducing the signals for IL-12 induction.

Dietary supplementation with *L. rhamnosus* HN001 has been shown to increase NK cell number in humans [56] and furthermore *L. casei* Shirota-fermented milk enhances cytotoxic activity of NK cells to target K562 cells [57]. Although IL-12 secreted by probiotic-stimulated monocytes activated NK cells in peripheral blood mononuclear cells (PBMCs) *in vitro* [57], it is unclear whether orally administered probiotics can induce IL-12 *in vivo* in the same way.

There is accumulating evidence showing that dietary supplementation with probiotics augments innate immune functions including phagocytic activity of neutrophils and cytotoxic activity of NK cells [56]. The activation of neutrophils and NK cells might be closely connected with the anti-infectious or anticancer abilities of probiotics. However, excessive activation of innate immunity by probiotics, particularly the documented triggering of IL-12 production, might entail the risk of aggravating allergy or autoimmune disease. Contrary to expectations, much research suggests that probiotics can improve disease symptoms in allergy or autoimmune diseases in human clinical trials and animal experiments. Therefore, a credible view of the effects of probiotics is that they do not augment immune responses unilaterally but rather redress the imbalances of a disordered immune system.

Currently there is little information on the host receptors and corresponding ligands of probiotics that lead to IL-

10 production. In general, the abilities of lactobacilli to elicit IL-10 production from human DCs and PBMCs are weaker than those of bifidobacteria [58,59]. That said, the addition of some *Lactobacillus* strains at high doses could induce high levels of IL-10, resulting in a decrease of IL-12 production in murine macrophage or DC cultures [51,60]. The relative abilities of probiotic strains to induce IL-12 and IL-10 are likely to be of clinical importance. In this regard, a recent report demonstrated a correlation between probiotic strains that trigger a higher IL-10:IL-12 ratio production from human PBMCs with the protective effects in a murine TNBS (2,4,6-trinitrobenzene sulfonic acid)-induced colitis model [61].

Probiotics might alsoexert their anti-inflammatory activity by inhibiting the secretion of inflammatory cytokines such IL-6 and IL-8. The polysaccharide–peptidoglycan complex of *L. casei* Shirota inhibited IL-6 production by lipopolysaccharide (LPS)-stimulated PBMCs from UC patients and murine large intestinal LP lymphocytes from mice with chronic colitis [62]. Furthermore, live but not heat-killed *L. casei* DN-114 001 cells could effectively downregulate spontaneous secretion of TNF-α by the inflamed mucosa of CD patients [63]. Similarly, Caco-2 cells pretreated with live and heat-killed *L. rhamnosus* GG secreted much lower levels of IL-8 after stimulation with TNF-α [64]. DNA from *Bifidobacterium* strains in VSL#3 could limit overproduction of proinflammatory cytokines in epithelial HT-29 cells induced by various proinflammatory stimuli [65]. Although the precise mechanisms of these anti-inflammatory effects are unknown, it has been proposed that probiotics suppress the NF-κB activation pathway by inhibiting the degradation of IκB in IECs, resulting in the inhibition of inflammatory responses in the intestinal mucosa [64,65].

## Regulation of T-cell functions by probiotics

Several *in vitro* studies have shown that probiotics can modulate the Th1:Th2 balance toward Th1 dominance indirectly through stimulating monocytes, macrophages or DCs. For example, *L. casei* Shirota has been shown to promote the development of Th1 cells through secretion of IL-12 by macrophages [66]. Furthermore, *L. rhamnosus* GG inhibited Th2 cytokine production by PBMCs from allergic patients through induction of IL-12 [67], and certain *Bifidobacterium* strains inhibited Th2 cytokine production through secretion of IL-10 by monocytes [68].

A large body of data now demonstrates that Treg cells are critically involved in controlling immunopathology in a wide variety of inflammatory diseases, and therefore, much attention has been paid recently to the abilities of probiotics to induce the development of these cells (also see the article by Tsuji and colleagues in this issue). For instance, *L. reuteri* ASM20016 and *L. casei* NIZO B255 have been shown *in vitro* to stimulate DC-SIGN on human monocyte-derived immature DCs and to trigger their differentiation into regulatory DCs; these cells were then able to induce IL-10–producing Treg cells [48]. In addition, *B. bifidum* W23 enhanced the differentiation of immature DCs derived from human cord blood monocytes into regulatory DCs and then induced appearance of IL-10–producing Treg cells *in vitro* [44]. It has also been proposed that probiotics are able to induce Treg cells through their stimulation of IECs. Indeed, certain *Lactobacillus* strains can stimulate Caco-2 cells to secrete transforming growth factor β (TGF-β) as well as thymic stromal lymphopoietin (TSLP), and these factors together promote the differentiation of immature DCs into regulatory DCs, which in turn induce TGF-β–secreting Treg cells [69]. The anti-inflammatory cytokine TGF-β is known to be important for the development and function of many types of Treg cells [70] (also see the article by Stein-Streilein in this issue). Recent evidence has shown that Treg cells induced by probiotics might play a pivotal role in controlling inflammatory diseases and in particular those associated with the gut. Indeed, in a murine TNBS-induced colitis model, dietary supplementation with VSL#3 ameliorated symptoms by inducing TGF-β–expressing Treg cells [71]. Further evidence for the role of probiotics in the induction of Treg cells and the control of inflammation has come from work with *L. rhamnosus* GG and *B. lactis* Bb-12. This study showed that dietary supplementation with these probiotic strains could induce TGF-β–secreting Treg cells, which correlated with suppression of the allergic symptoms in a model of murine asthma [72].

Probiotics therefore have the potential to induce both proinflammatory Th1 responses through their activation of IL-12 and anti-inflammatory responses by eliciting Treg cell development. This presents a dichotomy that might be explained by differences in the probiotic strains. Alternatively, different administration routes of probiotics might elicit different immune responses. Although parenteral injection or addition into *in vitro* culture might reveal a potential to induce Th1 responses, orally administered probiotics might trigger immune-modulating activities by promoting the development of Treg cells within the unique environment of the GALT.

Recently, IL-17–producing T helper cells (Th17 cells) have been regarded as crucial for the pathogenesis of several chronic inflammatory diseases such as multiple sclerosis and IBD. Th17 cells are abundant in the intestine, and the development of these cells seems to be regulated by $CD11b^+$DCs and macrophages in the LP [73]. Thus, orally administered probiotics could affect the development of Th17 cells and ameliorate clinical symptoms. Regulation of Th17 cell functions might be the next target for probiotic modulation of the mucosal immune system. In sum, probiotics could potentially play a role in the control of the entire immune network by affecting diverse sets of immune-regulatory cells in the intestine (see Figure 2).

## Caveats and future aspects of probiotics

Selecting suitable probiotic strains for the prevention or treatment of various diseases is of high importance, because the abilities of probiotics to modulate the immune system are strain dependent. Moreover, probiotics are used as either single strains or mixtures of strains. Although a mixture of probiotics is given on the assumption that might act in additive or synergistic ways, we need to apply caution when choosing and making such combinations. For instance, the abilities of *L. casei* CHCC3137 to induce IL-12 and upregulate co-stimulatory molecules on murine

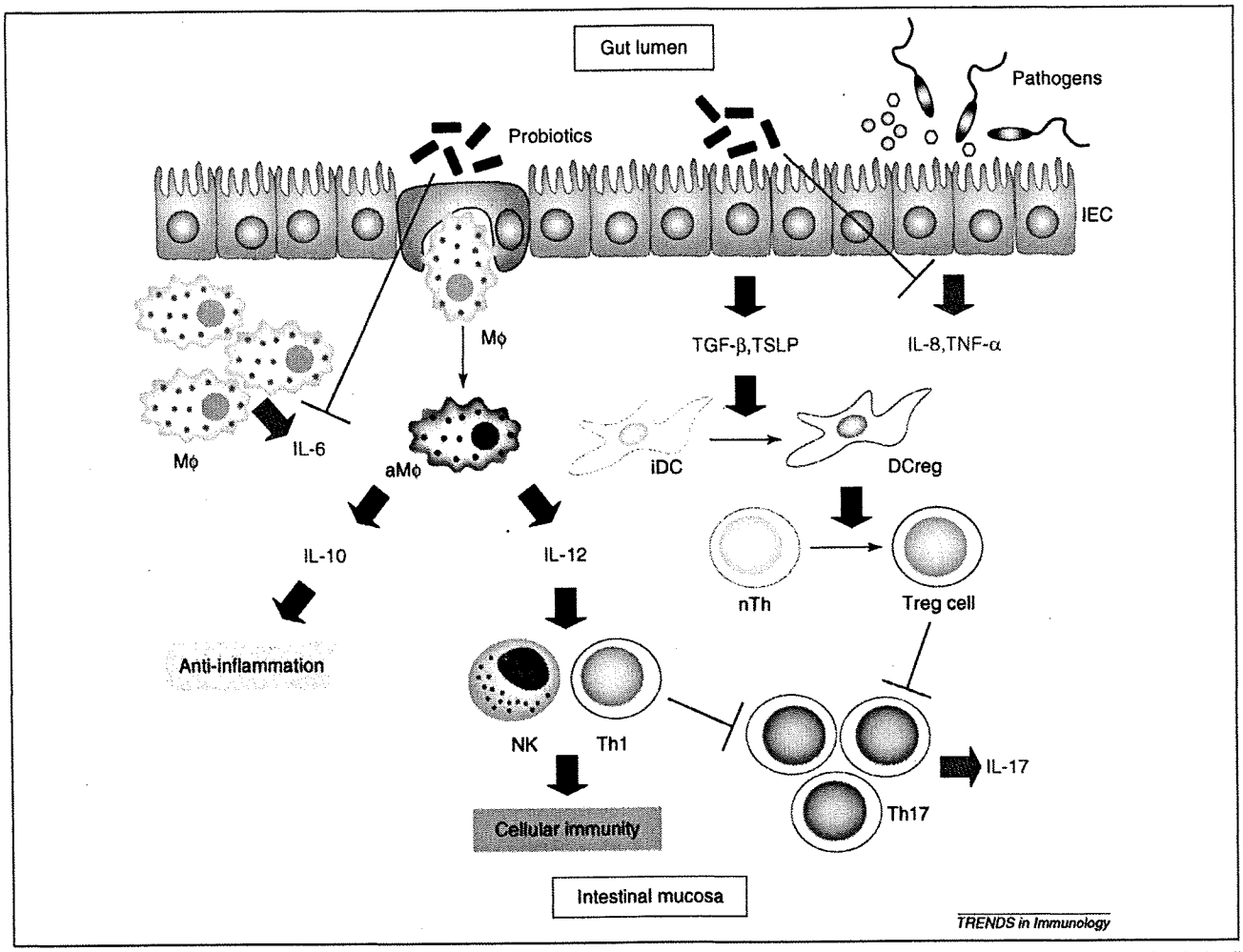


**Figure 2.** Supposed immune cell circuits in the intestine modulated by probiotics. While intestinal epithelial cells (IECs) exposed to pathogenic microbes or related stimuli produce proinflammatory mediators such as interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α), probiotics suppress the production of these cytokines and instead induce anti-inflammatory mediators such as transforming growth factor β (TGF-β) and thymic stromal lymphopoietin (TSLP), which can promote the differentiation of immature dendritic cells (iDCs) to regulatory dendritic cells (DCregs). Probiotics can also directly trigger the differentiation of DCregs, followed by induction of regulatory T cells (Tregs). Treg cells exert anti-inflammatory functions through their control of Th1, Th2 and probably Th17 cells. Macrophages (Mϕs) in the inflamed mucosa produce high amounts of IL-6, and probiotics can decrease their IL-6 production and increase IL-10 production. Gut Mϕs usually produce little IL-12, but probiotics might have the potential to enhance their IL-12 production depending on the surrounding environment. It is possible that probiotic-induced IL-12 promotes the differentiation of naive $CD4^+$ T cells (nThs) into Th1 cells, which augments natural killer (NK) cell activity, resulting enhancement of cytolytic functions as immune defense within the gut-associated lymphoid tissue (GALT). A novel Th subset, Th17 cells, are relatively enriched in the intestinal mucosa and involved in the exacerbation of autoimmune disease. Treg cells and Th1 cells can both inhibit the activity of Th17 cells. Taken together, probiotics can suppress excessive inflammatory responses and recruit anti-inflammatory cells such as Treg cells, leading to recovery and maintenance of homeostasis of intestinal mucosa.

DCs *in vitro* are masked by addition of another *Lactobacillus* strain [60]. This cross-regulatory effect of certain probiotic strains is also evident in another study showing that the efficacy of a mixture composed of *L. rhamnosus* GG and three other probiotic strains was weaker than that of *L. rhamnosus* GG alone in the treatment of infant AD [74]. The corollary of this is that *in vitro* cell culture experiments might well demonstrate the immune-modulating potential of a particular probiotic yet show wholly different results when supplemented in the diet where it would be impacted by the presence of abundant commensal bacteria. Therefore, probiotics might exhibit different effects depending on an individual's particular gut microflora. Recently, information on the responses of DCs and macrophages to simultaneous triggering of different TLRs or other pattern recognition receptors is gradually increasing and revealing hitherto unknown complexities. Decoding these pathways should help us to develop both more efficacious probiotic mixtures and future probiotics tailor-made to an individual host's gut microflora. Finally, the unfortunate outcome of a recent clinical trial has highlighted the importance of the route of administration of probiotics (see Box 2).

Our modern environment is rapidly changing, and it is becoming increasingly important to devise a suitable lifestyle to adapt ourselves to these changes. As discussed above, probiotics might be one useful tool for maintaining health, but it is imperative that these are determined according to an evidence-based approach informed by their immune-modulating abilities. Because probiotics are essentially foods, prophylaxis of disease rather than its treatment is one of the more compelling promises offered by them.

**Box 2. The importance of choice and administration route of probiotics for clinical application**

A recent study using probiotics has dramatically highlighted some important caveats associated with their clinical application. This study investigated whether a probiotic mixture (three *Lactobacillus* strains, two *Bifidobacterium* strains and one *Lactococcus* strain) administered to severe acute pancreatitis patients could prevent the incidence of opportunistic infection and improve clinical symptoms. The probiotics showed no significant impact on the incidence of opportunistic infections but more worryingly showed a higher mortality in the probiotic-treated group [79]. However, it should be noted that the probiotics used in this study were transfused directly into the ileum of patients, which is an administration route totally different from that of other clinical trials using probiotics. Nevertheless, this observation underscores the fact that, under certain conditions, probiotics might not be wholly without risk, and the selection of safe but effective probiotic strains will be crucial for their widespread clinical application.

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IMMUNOLOGY ORIGINAL ARTICLE

# Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms

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## Summary

We previously showed that *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion can stimulate macrophages to induce large a quantity of interleukin-12 (IL-12). In this study, we examined the influence of lactobacilli and bacterial cell wall components on IL-12 production by macrophages that was induced by *Lactobacillus casei*, which has a rigid cell wall. Easily digestible lactobacilli such as *Lactobacillus johnsonii* and *Lactobacillus plantarum* or their intact cell walls (ICWs) weakly or very weakly induced IL-12 production by macrophages, and inhibited *L. casei*-induced IL-12 production. While the ICW of *L. casei* was resistant to intracellular digestion and did not inhibit *L. casei*-induced IL-12 production, its polysaccharide-depleted ICW, i.e. intact peptidoglycan, was sensitive to intracellular digestion and inhibited *L. casei*-induced IL-12 production. Furthermore, the peptidoglycans of *L. johnsonii*, *L. plantarum* and *Staphylococcus aureus* also inhibited *L. casei*-induced IL-12 production. Peptidoglycans from lactobacilli suppressed *L. casei*-induced expression of IL-12p40 but not IL-12p35 mRNA. Inhibition of IL-12 production by peptidoglycan was mitigated in Toll-like receptor 2 (TLR2)-deficient macrophages compared with the inhibition in wild-type macrophages. A derivative of the minimal structural unit of peptidoglycan (6-*O*-stearoyl-muramyl dipeptide) recognized by nucleotide-binding oligomerization domain 2 (NOD2) could also suppress *L. casei*-induced IL-12 production. These findings demonstrate that easily digestible bacteria and peptidoglycan suppress IL-12 production through pattern recognition receptors such as TLR2 and NOD2. IL-12 production in the gut may be negatively regulated by the simultaneous inhibitory actions of various resident bacteria that are susceptible to intracellular digestion.

**Keywords:** interleukin-12; intracellular digestion; lactobacilli; peptidoglycan; Toll-like receptor 2

doi:10.1111/j.1365-2567.2009.03095.x
Received 4 November 2008; revised 8 January 2009; accepted 26 February 2009.
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## Introduction

Lactobacilli are members of the indigenous intestinal microflora in humans, and they are currently recognized as the most popular probiotics, i.e. live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[1] Of the several health-promoting effects of probiotic lactobacilli, their immunoregulatory functions have been studied with the greatest interest.[2,3] Some strains of lactobacilli stimulate macrophages and dendritic cells to secrete higher amounts of interleukin-12 (IL-12), which elicits the innate immune response, and thereby the incidence of infectious diseases and cancers decreases.[4,5] The IL-12-inducing ability of *Lactobacillus* strains is now considered as a critical index in the selection of immunostimulatory probiotic strains. However, dysregulated overproduction of IL-12 and the related cytokine IL-23, which shares the p40 subunit with IL-12, causes some types of inflammation.[6,7] Therefore, the production of IL-12 must be tightly regulated to maintain homeostasis in the host.


 

Please cite this article in press as: Shida K. *et al.* Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms, Immunology (2009) doi: 10.1111/j.1365-2567.2009.03095.x

IL-12 is a heterodimeric protein composed of two disulphide-linked subunits, namely, p35 and p40. The regulation of IL-12 expression is a complex process, because the genes encoding these subunits are located on different chromosomes, and their expression is differentially regulated by various stimuli. Recent reviews[5,8] provide good, detailed summaries of the regulation of IL-12 expression. (i) The expression of p35 and p40 involves the activation of nuclear factor (NF)-$\kappa$B, and in particular c-Rel, which is dependant on a myeloid differentiation primary response gene 88 (MyD88)-mediated signalling pathway, after Toll-like receptors (TLRs) are stimulated by microbial components. (ii) The Toll/IL-1 receptor-domain-containing adaptor protein inducing interferon (IFN)-$\beta$ (TRIF)-mediated pathway activates IFN regulatory factor 3 (IRF3) and enhances p35 expression both directly and indirectly by inducing the secretion of IFN-$\beta$. Nucleotide-binding oligomerization domain 2 (NOD2) is a cytoplasmic receptor for the by-products of peptidoglycan digestion; the effect of NOD2 stimulation on IL-12 production is a subject of controversy. Tada *et al.*[9] demonstrated the synergistic effect of muramyl dipeptide (MDP), a NOD2 ligand, with that of the TLR3, TLR4 or TLR9 ligand on the induction of IL-12 production by human dendritic cells, while Watanabe *et al.*[10] revealed that MDP inhibited IL-12 production by murine macrophages. Moreover, a recent study showed that certain strains of lactobacilli that have weak IL-12-inducing ability inhibit the IL-12 production that is potently induced by some other strains in human and murine dendritic cells.[11,12] However, the precise mechanisms of this inhibitory effect have not yet been elucidated. Taken together, these findings suggest that IL-12 production is tightly regulated through various mechanisms, probably because of the pivotal biological functions of this cytokine.

Recently, we showed that, for *Lactobacillus* strains to induce IL-12 production, it is critical for their cell walls to be resistant to intracellular digestion.[13] *Lactobacillus* strains that have a rigid cell wall that is resistant to intracellular digestion, such as *Lactobacillus casei* and *Lactobacillus rhamnosus*, potently induced IL-12 production by mouse peritoneal macrophages, whereas those strains that have an easily digestible cell wall, such as *Lactobacillus johnsonii*, hardly induced IL-12 production. Our results also revealed that *L. casei* induces IL-12 production through TLR2-independent mechanisms and that the insoluble intact cell wall (ICW) and not the solubilized cell wall of this strain is responsible for inducing IL-12 production. These findings strongly suggest that the ICW structure rather than soluble cell wall components of lactobacilli is essential for the effective induction of IL-12 production, at least in the case of mouse peritoneal macrophages. Sekine *et al.*[14] examined the antitumour effects of three kinds of morphologically distinct cell wall preparations isolated from *Bifidobacterium infantis* and showed that the antitumour activity of the cell wall preparation increases in proportion to the degree of integrity of its physical form. The potent antitumour activity of the cell wall preparation having physical integrity of the cell wall structure, i.e. ICW, might be related to its potent ability to induce IL-12 production.

In the present study, we further investigated the IL-12 production by macrophages stimulated with a combination of *L. casei* and other strains possessing easily digestible cell walls. The data obtained showed that the latter strains inhibited *L. casei*-induced IL-12 production and that the peptidoglycan in the cell walls of these strains was responsible for this inhibitory effect. This study reveals a novel mechanism by which peptidoglycan from *Lactobacillus* cell walls mediates the negative regulation of IL-12 production.

## Materials and methods

### *Reagents and culture medium*

Trypsin and cytochalasin D were purchased from Sigma (St Louis, MO). *N*-acetylmuramidase SG isolated from *Streptomyces globisporus* was obtained from Seikagaku Corp. (Tokyo, Japan). Benzon nuclease and pronase were purchased from Merk (Darmstadt, Germany) and Roche Diagnostics (Indianapolis, IN), respectively. Purified insoluble peptidoglycan from *Staphylococcus aureus*, MDP, 6-*O*-stearoyl-MDP (L18-MDP), and lauroyl-$\gamma$-D-glutamyl-meso-diaminopimelic acid (C12-DAP) were purchased from InvivoGen (San Diego, CA). RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 0·05 mM 2-mercaptoethanol was used to culture macrophages.

### *Animals*

Female BALB/c mice and male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Male TLR2-deficient mice with a C57BL/6 genetic background were obtained from Oriental Bioservice (Kyoto, Japan). Animals aged between 8 and 12 weeks were used. All the experiments were performed in accordance with the guidelines for the care and use of laboratory animals set by Yakult Central Institute.

### *Bacteria*

Heat-killed lactobacilli were used in this study, unless otherwise specified. On the basis of its resistance to gastric juices and bile acid and its ability to survive in the gastrointestinal tract, the probiotic strain *L. casei* strain Shirota (YIT 9029) was originally isolated at Yakult Central Institute. *Lactobacillus johnsonii* JCM 2012$^T$ and

*Lactobacillus plantarum* ATCC 14917$^T$ were obtained from the Japan Collection of Microorganisms (Wako, Japan) and American Type Culture Collection (Rockville, MD), respectively. The bacteria were cultured at 37° for 20 hr in lactobacilli-de Man, Rogosa and Sharpe (MRS) broth (Difco, Detroit, MI), washed with sterile distilled water, heated at 100° for 30 min, and then lyophilized.

Fluorescein isothiocyanate (FITC)-labelled lactobacilli and cell wall components were prepared using FITC isomer-I (Dojindo Lab., Kumamoto, Japan), as described previously.[13]

### *Preparation of cell wall components*

Cell wall components were obtained from heat-killed lactobacilli, and the preparation steps are summarized in Fig. 1. ICW was obtained using the method of Sekine *et al.*[14] with minor modifications.[13] Briefly, heat-killed cells were boiled in 0·3% sodium dodecyl sulphate (SDS) solution for 15 min and washed with acetone. The cells were treated with pronase, and delipidated by successive refluxing with methanol, methanol–chloroform–water (1 : 1 : 1), and methanol–chloroform (1 : 1). The delipidated preparation was treated with benzon nuclease and pronase. The insoluble material was washed with distilled water and lyophilized; the resultant product was used as the ICW preparation. To obtain intact peptidoglycan and cell wall polysaccharides (PS), ICW was treated with 47% hydrogen fluoride at 4° for 20 hr, whereby the polysaccharide moiety was removed from the linkage region of ICW. The phosphodiester bonds were selectively cleaved under these conditions.[15] After centrifugation, the precipitate was washed with distilled water and used as the intact peptidoglycan preparation. It was likely that the peptidoglycan preparation was free of contaminants such as lipoteichoic acid (LTA), wall teichoic acid and nucleic acid, as phosphorus was below the level of detection (< 10 nmol/mg) in the preparation by the method of Lowry *et al.*[16] The supernatant was neutralized with 10 M NaOH, dialysed against distilled water, lyophilized, and used as the PS preparation. Soluble peptidoglycan (sPGN) was prepared by exhaustive digestion of intact peptidoglycan by *N*-acetylmuramidase treatment for 16 hr. The digested material solution was then treated with trypsin, dialysed against distilled water, lyophilized, and used as the sPGN preparation.

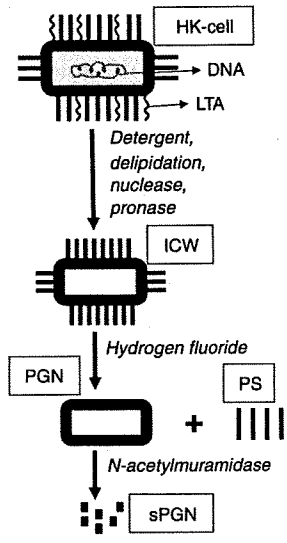


Figure 1. Schematic representation of preparation steps for cell wall components. HK-cell, heat-killed lactobacillus cell; LTA, lipoteichoic acid; ICW, intact cell wall; PGN, intact peptidoglycan; PS, peptidoglycan-associated polysaccharide; sPGN, solubilized peptidoglycan.

### *Isolation and culture of peritoneal macrophages*

Peritoneal macrophages were isolated from BALB/c mice, unless otherwise specified. Mice were intraperitoneally injected with 2 ml of 4% thioglycollate broth (Difco). After 4 days, peritoneal macrophages were isolated as described previously.[13] For the analysis of cytokine secretion, peritoneal macrophages ($1 \times 10^5$ cells) were cultured in a 96-well culture plate (Nunc, Roskilde, Denmark) with heat-killed lactobacilli or their cell wall components (1–30 μg/ml) in the presence or absence of heat-killed *L. casei* (10 μg/ml) in 200 μl of RPMI-1640 medium. The supernatants of these cultures were collected at 24 hr to determine the levels of IL-12p70, tumour necrosis factor-α (TNF-α), and IL-10.

### *Analysis of IL-12 mRNA expression*

Peritoneal macrophages ($1{\cdot}2 \times 10^6$ cells) were cultured in a 12-well culture plate (Nunc) with peptidoglycan (10 μg/ml) in the presence or absence of heat-killed *L. casei* (10 μg/ml) in 2·4 ml of RPMI-1640 medium. Total RNA was extracted from the cells at the indicated time-points (0–24 hr) using the RNAqueous kit (Ambion, Austin, TX), and reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (PCR) was performed using TaqMan Universal PCR master mix and TaqMan gene expression assays for IL-12p35 and IL-12p40 expression (Applied Biosystems) in an ABI 7500 Real Time PCR System (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control to normalize the expression of the IL-12 genes.

### *Light microscopic analysis of macrophages to observe intracellular digestion*

Peritoneal macrophages ($2 \times 10^5$ cells) were plated onto round, 12-mm collagen type I-coated cover glasses (Asahi



 

Techno Glass, Tokyo, Japan) and cultured in a 24-well culture plate (Nunc) in 1·2 ml of RPMI-1640 medium. After preincubation of the culture for 16 hr, heat-killed lactobacilli were introduced at a concentration of 10 μg/ml. The macrophages were harvested after 24 hr, washed with phosphate-buffered saline (PBS), fixed with methanol for 10 min, and then stained with Giemsa (Sigma). Intracellular digestion of bacteria was observed under a BH-2 light microscope (Olympus, Tokyo, Japan).

*Confocal laser scanning microscope analysis of macrophages to observe intracellular digestion*

Peritoneal macrophages ($2 \times 10^5$ cells) on round, 12-mm collagen type I-coated cover glasses were cultured with FITC-labelled lactobacilli or cell wall components (10 μg/ml) in a 24-well culture plate in 1·2 ml of RPMI-1640 medium. The macrophages were harvested after 24 hr, washed with PBS, and fixed with methanol. After the plasma membrane and the nucleus had been stained with phycoerythrin (PE)-conjugated rat anti-mouse CD11b monoclonal antibody (BD PharMingen, San Diego, MO) and TO-PRO-3 dye (Molecular Probes, Eugene, OR), respectively, the macrophages were observed and photographed with a LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

*Flow cytometric analysis of phagocytosis*

Peritoneal macrophages ($6 \times 10^5$ cells) were cultured in a 24-well culture plate with FITC-labelled lactobacilli (10 μg/ml) in 1·2 ml of RPMI-1640 medium for 24 hr. The macrophages were dislodged by treating them with 10 mM ethylenediaminetetraacetic acid (EDTA)/PBS for 10 min, and they were then washed with 3 mM EDTA/PBS. Flow cytometric analysis was performed using an EPICS Altra flow cytometer equipped with EXPO32 software (Beckman Coulter, Miami, FL). In order to investigate whether peptidoglycan inhibits phagocytosis of *L. casei* by macrophages, macrophages were cultured with FITC-labelled *L. casei* (10 μg/ml) in the presence or absence of peptidoglycan (10 μg/ml) and cytochalasin D (5 μg/ml) for 16 hr, and analysed by flow cytometry.

*N-acetylmuramidase treatment*

The sensitivity of lactobacilli and their cell wall components to *N*-acetylmuramidase was evaluated on the basis of the decrease in their optical density at 600 nm (OD600), as described previously.[13] In brief, heat-killed lactobacilli or their cell wall components suspended at a concentration of 2 mg/ml in 50 mM Tris–maleate buffer (pH 7·0) containing 4 mM $MgCl_2$ were treated with *N*-acetylmuramidase (10 μg/ml) for 10, 30, 60 and 120 min. After heat-denaturation of the enzyme, the test materials were further treated with 2% SDS to dissolve the protoplasts formed as a result of cell wall digestion. The OD600 values were determined and expressed as a percentage of the OD600 values before enzyme treatment.

*Enzyme-linked immunosorbent assay (ELISA) for detection of cytokines*

A sandwich ELISA was used to determine the levels of IL-12p70, IL-10 and TNF-α in the supernatants of the cultures. Rat anti-mouse IL-12 (clone 9A5) and IL-10 (clone JES5-SXC1) monoclonal antibodies were used as the capture antibodies, and the corresponding detection antibodies for these were biotinylated rat anti-mouse IL-12 (clone C17.8) and IL-10 (clone JES5-2A5) monoclonal antibodies. These antibodies and recombinant mouse IL-12 and IL-10 were purchased from BD PharMingen. Mouse TNF-α Duoset (Genzyme, Cambridge, MA) was used to determine the TNF-α levels in the cultures.

## Results

### Sensitivity of lactobacilli to digestion

The results of our previous study showed that the phagocytosis and subsequent digestion of lactobacilli by macrophages are closely related to IL-12 production and that the sensitivity of *Lactobacillus* strains to digestion with *N*-acetylmuramidase is negatively correlated to their ability to induce IL-12 production.[13] Therefore, we initially tested the *in vitro* sensitivity of heat-killed *L. casei*, *L. johnsonii* and *L. plantarum* to *N*-acetylmuramidase. While *L. casei* was resistant to enzyme treatment, *L. johnsonii* and *L. plantarum* were sensitive (Fig. 2a). Digestion of these three strains by mouse peritoneal macrophages was also examined. Light microscopic analysis revealed that, while *L. casei* was hardly digested at all after 24 hr of macrophage culture, *L. johnsonii* was easily digested (Fig. 2b,c). Some of the *L. plantarum* cells that were phagocytosed by macrophages seemed to retain their cell morphology during the culture period (Fig. 2d). Confocal laser scanning microscope analysis using FITC-labelled lactobacilli clearly showed that *L. casei* was hardly digested at all by the macrophages, *L. johnsonii* was easily digested, and *L. plantarum* showed an intermediate pattern between the former two strains (Fig. 2e–g). Flow cytometric analysis confirmed that all these strains were phagocytosed well by macrophages (Fig. 2h–j).

### IL-12 production by macrophages stimulated with a single *Lactobacillus* strain or a combination of two strains

Macrophages were cultured with heat-killed *L. casei*, *L. johnsonii* or *L. plantarum* for 24 hr, and the

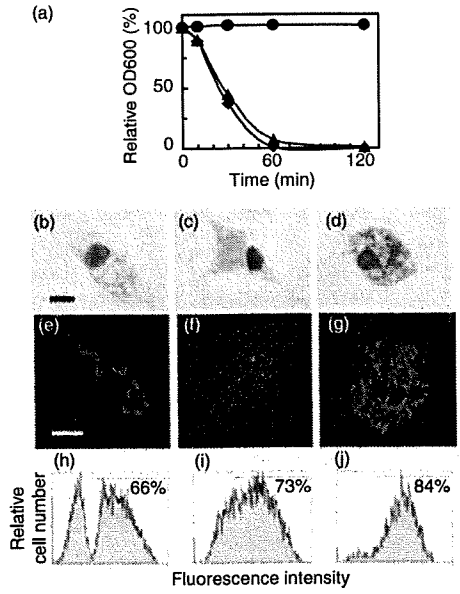


Figure 2. Digestion of lactobacilli by *N*-acetylmuramidase treatment or macrophages. (a) *Lactobacillus casei* (circles), *Lactobacillus johnsonii* (triangles) and *Lactobacillus plantarum* (diamonds) were treated with *N*-acetylmuramidase for 10, 30, 60 and 120 min, and the extent of digestion of the cell wall was evaluated using the reduction in optical density at 600 nm (OD600). Peritoneal macrophages were cultured with unlabelled (b–d) or fluorescein isothiocyanate (FITC)-labelled (e–j) *L. casei* (b, e), *L. johnsonii* (c, f) and *L. plantarum* (d, g) for 24 hr. Intracellular digestion of lactobacilli was observed using a light microscope after staining with Giemsa (b–d) or a confocal laser scanning microscope after staining with phycoerythrin-conjugated anti-CD11b antibody and TO-PRO-3 dye (e–g). Bars, 5 μm. Phagocytosis of *L. casei* (h), *L. johnsonii* (i) and *L. plantarum* (j) was analysed by flow cytometry. The percentage of fluorescence-positive cells is shown.

concentrations of IL-12p70, TNF-α and IL-10 in the supernatants were measured by ELISA. As shown in Fig. 3, *L. casei* potently induced IL-12 production in a dose-dependent manner at the tested doses (1–30 μg/ml), whereas *L. plantarum* weakly induced IL-12 production at an optimal dose of 3 μg/ml. *Lactobacillus johnsonii* hardly induced any IL-12 production. The induction pattern of TNF-α production was similar to that of IL-12, except that *L. johnsonii* induced similar levels of TNF-α to *L. plantarum*. In contrast, IL-10 production was strongly induced by *L. plantarum* and weakly induced by *L. casei*, especially at low doses. As was the case for IL-12 production, *L. johnsonii* hardly induced any IL-10 production.

Next, macrophages were cultured with these three strains along with 10 μg/ml heat-killed *L. casei*, and the effects of the strains on *L. casei*-induced cytokine production were examined. *Lactobacillus casei*-induced IL-12 production was suppressed in a dose-dependent manner by the addition of *L. johnsonii* and *L. plantarum* (Fig. 3).

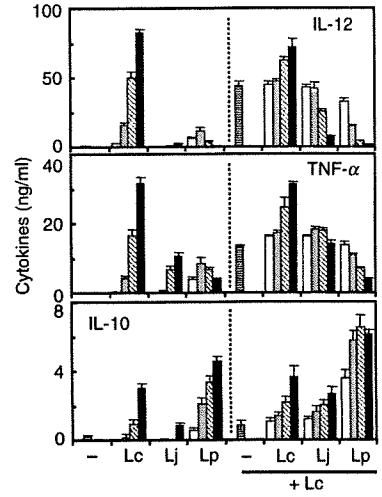


Figure 3. *Lactobacillus johnsonii* and *Lactobacillus plantarum* inhibit *Lactobacillus casei*-induced interleukin-12 (IL-12) production. Peritoneal macrophages were cultured with *L. casei* (Lc), *L. johnsonii* (Lj) and *L. plantarum* (Lp) at concentrations of 1 (white), 3 (grey), 10 (hatched) and 30 (black) μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12, tumour necrosis factor-α (TNF-α), and IL-10 in culture supernatants were determined by enzyme-linked immunosorbent assay. Data are the mean ± standard deviation for triplicate cultures. Experiments were repeated three times with similar results; –, no additives or *L. casei* only.

*Lactobacillus casei*-induced TNF-α production was inhibited only in the presence of *L. plantarum*, and the induction of IL-10 production was not inhibited by either of the strains.

When live lactobacilli were used in these experiments, *L. casei* was resistant but *L. johnsonii* and *L. plantarum* were sensitive to *N*-acetylmuramidase digestion, and only *L. casei* showed strong induction of IL-12 production; live *L. johnsonii* and *L. plantarum*, but not *L. casei*, inhibited *L. casei*-induced IL-12 production, as was observed in the case of the corresponding heat-killed cells (data not shown).

## ICW is responsible for the ability of lactobacilli to induce and inhibit IL-12 production

We previously showed that the insoluble ICW of *L. casei* is responsible for IL-12 production.[13] Therefore, we prepared ICWs from the three *Lactobacillus* strains and tested their ability to induce cytokine production; we also tested the ability of the ICWs to inhibit *L. casei*-induced cytokine production. Similar to the corresponding intact heat-killed cells, the ICWs of *L. casei*, *L. johnsonii* and *L. plantarum* induced the production of IL-12, TNF-α and IL-10 (Fig. 4). Furthermore, the ICWs of *L. johnsonii* and *L. plantarum* inhibited the *L. casei*-induced production



 

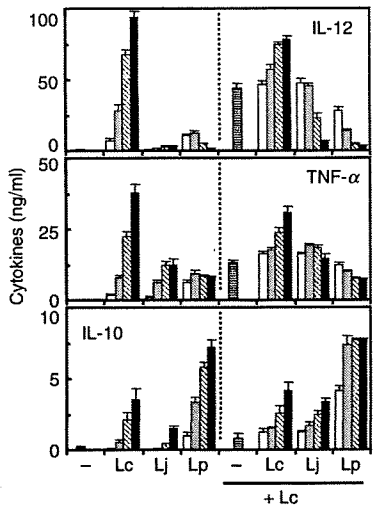


Figure 4. The intact cell wall is responsible for the ability of lactobacilli to induce and inhibit interleukin-12 (IL-12) production. Peritoneal macrophages were cultured with the intact cell walls of *Lactobacillus casei* (Lc), *Lactobacillus johnsonii* (Lj) and *Lactobacillus plantarum* (Lp) at concentrations of 1 (white), 3 (grey), 10 (hatched) and 30 (black) μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12, tumour necrosis factor-α (TNF-α) and IL-10 in culture supernatants were determined by enzyme-linked immunosorbent assay. Data are the mean ± standard deviation for triplicate cultures. Experiments were repeated three times with similar results; –, no additives or *L. casei* only.

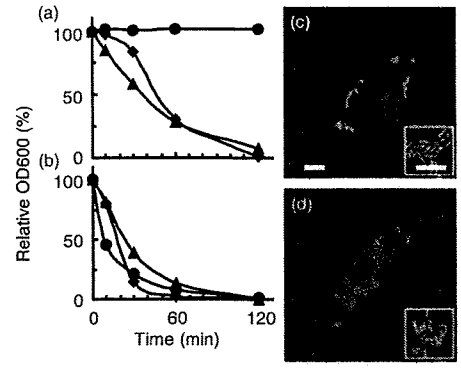


Figure 5. Intact peptidoglycan of *Lactobacillus casei* is sensitive to *N*-acetylmuramidase treatment and macrophage intracellular digestion. The intact cell walls (a) or intact peptidoglycans (b) of *L. casei* (circles), *Lactobacillus johnsonii* (triangles) and *Lactobacillus plantarum* (diamonds) were treated with *N*-acetylmuramidase for 10, 30, 60 and 120 min, and the extent of digestion of the cell wall components was evaluated using the reduction in optical density at 600 nm (OD600). Peritoneal macrophages were cultured with fluorescein isothiocyanate (FITC)-labelled intact cell wall (c) or intact peptidoglycan (d) of *L. casei* for 24 hr. Intracellular digestion of the cell wall components was observed using a confocal laser scanning microscope after staining with phycoerythrin-conjugated anti-CD11b antibody and TO-PRO-3 dye. Inserts are FITC-labelled cell wall components before addition to the cultures. Bars, 5 μm.

of IL-12, as was observed in the case of the corresponding intact cells. The ICW of *L. plantarum* also inhibited *L. casei*-induced TNF-α production, as did intact *L. plantarum* cells. These data suggest that the ICW is responsible for the ability of these strains to induce and inhibit IL-12 production.

### Intact peptidoglycan is sensitive to digestion

Our previous finding that the removal of the polysaccharide moiety from the ICW of *L. casei* resulted in the loss of its IL-12-inducing ability suggested that the polysaccharide-depleted ICW of *L. casei* might not be resistant to intracellular digestion.[13] We prepared polysaccharide-depleted ICWs, i.e. intact peptidoglycans, from the ICWs of the three strains by hydrogen fluoride treatment, and we examined their sensitivity to *N*-acetylmuramidase treatment and macrophage intracellular digestion. The intact peptidoglycan as well as ICW retained the rod-like morphology before being phagocytosed by macrophages (insets in Fig. 5c,d). The intact peptidoglycan, but not the ICW, of *L. casei* was susceptible to *N*-acetylmuramidase treatment (Fig. 5a,b). The ICWs of *L. johnsonii* and *L. plantarum* were sensitive to *N*-acetylmuramidase treatment, and removal of the polysaccharide moiety seemed to increase their sensitivity to *N*-acetylmuramidase treatment. Moreover, the intact peptidoglycan, but not the ICW, of *L. casei* that was phagocytosed by the macrophages rapidly lost its morphology, suggesting that it might be susceptible to intracellular digestion (Fig. 5c,d).

### Intact peptidoglycan inhibits *Lactobacillus casei*-induced IL-12 production

We evaluated the inhibitory effects of cell wall components obtained from *L. casei* on *L. casei*-induced IL-12 production. Only the intact peptidoglycan, not the ICW or the cell wall polysaccharides, had an inhibitory effect (Fig. 6a). The intact peptidoglycans of *L. johnsonii*, *L. plantarum* and *S. aureus* inhibited *L. casei*-induced IL-12 production but did not inhibit *L. casei*-induced TNF-α production; this result was different from that obtained for the intact cells and ICW of *L. plantarum* (Fig. 6b). The intact peptidoglycans of these strains inhibited the IL-12 production that was potently induced by other *Lactobacillus* strains having a rigid cell wall, such as *L. rhamnosus* (data not shown). We could not examine whether intact peptidoglycan inhibits IL-12 production induced by pathogenic stimuli, because any such stimuli tested (lipopolysaccharide, flagellin, heat-killed *S. aureus* and *Escherichia coli*) could not induce IL-12 production effectively in the peritoneal macrophage cultures used in

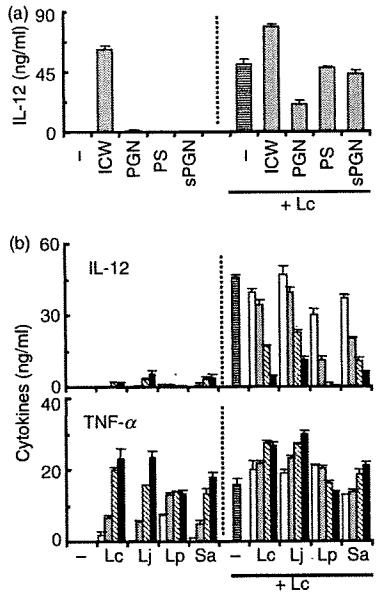


Figure 6. Intact peptidoglycan inhibits *Lactobacillus casei*-induced interleukin-12 (IL-12) production. (a) Peritoneal macrophages were cultured with the intact cell wall (ICW), intact peptidoglycan (PGN), cell wall polysaccharides (PS), and soluble peptidoglycan (sPGN) of *L. casei* at a concentration of 10 μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). (b) Peritoneal macrophages were cultured with the intact peptidoglycans of *L. casei* (Lc), *Lactobacillus johnsonii* (Lj), *Lactobacillus plantarum* (Lp) and *Staphylococcus aureus* (Sa) at concentrations of 1 (white), 3 (grey), 10 (hatched), and 30 (black) μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12 and tumour necrosis factor-α (TNF-α) in culture supernatants were determined by ELISA. Data are the mean ± standard deviation for triplicate cultures. Experiments were repeated three times with similar results; –, no additives or *L. casei* only.

this study. The solubilized form of the *L. casei* peptidoglycan did not effectively inhibit *L. casei*-induced IL-12 production, suggesting the importance of the recognition of intact peptidoglycan by the receptors on macrophages or of the steps involved in the phagocytosis of peptidoglycan (Fig. 6a).

## Peptidoglycan inhibits IL-12 production by reducing the IL-12p40 mRNA expression

We investigated whether peptidoglycan inhibits the expression of IL-12p35 and IL-12p40 mRNAs. The expression of IL-12 mRNAs in macrophages cultured with *L. casei* cells or the peptidoglycans of *L. casei* and *L. plantarum* was analysed. *Lactobacillus casei* cells induced the expression of both IL-12p35 and IL-12p40 mRNAs in the macrophages, with peak expression at 16 hr of culture; however, the peptidoglycans of *L. casei* and *L. plantarum* induced IL-12p35 mRNA expression more effectively than did *L. casei* cells, with peak expression observed at 8 hr of culture, but the peptidoglycans hardly induced any IL-12p40 mRNA expression (Fig. 7a,b). When macrophages were cultured for 8 and 16 hr with a combination of *L. casei* and the peptidoglycans of *L. casei* and *L. plantarum*, the peptidoglycans markedly inhibited the *L. casei*-induced expression of IL-12p40 but not IL-12p35 mRNA (Fig. 7c,d). These data suggest that the inhibition of IL-12 production by the peptidoglycans results from a reduction in IL-12p40 mRNA expression.

## Peptidoglycan inhibits IL-12 production by neither suppressing phagocytosis nor inducing IL-10 production

We determined whether peptidoglycan inhibits the phagocytosis of *L. casei* by macrophages because we had previously observed that the phagocytosis of *L. casei* is essential for it to induce IL-12 production.[13] Macrophages

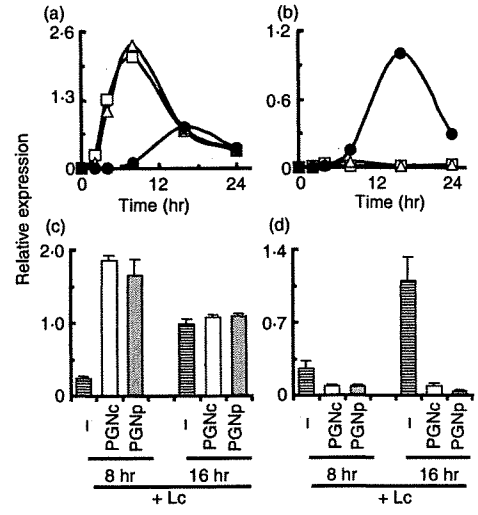


Figure 7. Inhibition of *Lactobacillus casei*-induced interleukin-12p40 (IL-12p40) gene expression by intact peptidoglycan. Peritoneal macrophages were cultured with *L. casei* (closed circles) and the intact peptidoglycans of *L. casei* (open triangles) and *Lactobacillus plantarum* (open squares) for 2, 4, 8, 16 and 24 hr, and the mRNA expression of IL-12p35 (a) and IL-12p40 (b) was analysed by quantitative reverse transcription–polymerase chain reaction. The relative expression of IL-12 genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. Experiments were repeated twice with similar results. In order to examine the effect of the intact peptidoglycans of *L. casei* (PGNc) and *L. plantarum* (PGNp) on *L. casei*-induced IL-12 mRNA expression, peritoneal macrophages were cultured with PGNc and PGNp at a concentration of 10 μg/ml in the presence (+ Lc) of *L. casei* (10 μg/ml) for 8 and 16 hr, and the mRNA expression of IL-12p35 (c) and IL-12p40 (d) was analysed. Data are the mean ± standard deviation for three independent experiments; –, *L. casei* only.



 

were cultured with FITC-labelled *L. casei* for 16 hr with or without unlabelled peptidoglycans or cytochalasin D, an inhibitor of actin polymerization; phagocytosis was then analysed by flow cytometry. We observed that the peptidoglycans of *L. casei* and *L. plantarum* did not interfere with the phagocytosis of *L. casei*, whereas cytochalasin D markedly suppressed it (Fig. 8a).

It is well known that IL-10 inhibits the secretion of IL-12 by macrophages.[5] Therefore, we used a neutralization antibody against IL-10 in order to determine whether IL-10 mediated the inhibition of IL-12 production by peptidoglycan. Macrophages were cultured with a combination of *L. casei* and cell wall components of *L. casei* and *L. plantarum* in the presence of the anti-IL-10 or control antibody. As shown in Fig. 8b, the ICW of *L. plantarum* induced high amounts of IL-10 and inhibited *L. casei*-induced IL-12 production in the absence of the anti-IL-10 antibody. Addition of the anti-IL-10 antibody abrogated the inhibitory effect of the *L. plantarum* ICW on IL-12 production, suggesting that IL-10 mediated the inhibition of IL-12 production by *L. plantarum* ICW. Moreover, the ICW of *L. plantarum* could effectively induce IL-12 production in the presence of the anti-IL-10 antibody. In contrast, the inhibition of IL-12 production by the peptidoglycans of *L. casei* and *L. plantarum* was not affected by the addition of the anti-IL-10 antibody, suggesting that peptidoglycan inhibits *L. casei*-induced IL-12 production through IL-10-independent mechanisms.

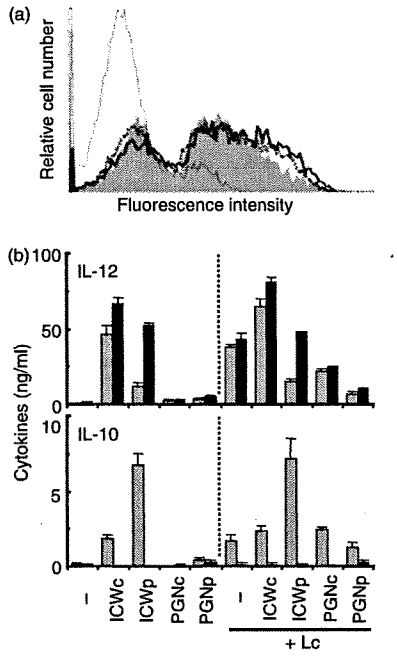


Figure 8. Inhibition of *Lactobacillus casei*-induced interleukin-12 (IL-12) production by intact peptidoglycan is mediated by neither interfering with phagocytosis of *L. casei* nor inducing IL-10 production. (a) Peritoneal macrophages were cultured with fluorescein isothiocyanate-labelled *L. casei* in the absence (shaded) or presence of the intact peptidoglycans of *L. casei* (thick line) and *Lactobacillus plantarum* (thick dotted line) or cytochalasin D (thin line) for 16 hr, and analysed by flow cytometry. Experiments were repeated twice with similar results. (b) Peritoneal macrophages were cultured with the intact cell walls (ICWc and ICWp) or intact peptidoglycans (PGNc and PGNp) of *L. casei* and *L. plantarum* at a concentration of 10 μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. Anti-IL-10 (black) or isotype control (grey) antibody (10 μg/ml) was added at the beginning of culture. The levels of IL-12 and IL-10 in culture supernatants were determined by enzyme-linked immunosorbent assay. Data are the mean ± standard deviation for triplicate cultures. Experiments were repeated twice with similar results; –, antibody only or *L. casei* plus antibody.

## TLR2 is involved in the inhibition of IL-12 production by peptidoglycan

Using macrophages isolated from TLR2-deficient mice, we examined whether the recognition of peptidoglycan by TLR2 is involved in the inhibition of IL-12 production. IL-12 production by both TLR2-deficient and wild-type macrophages was induced by stimulation with *L. casei*, suggesting that the recognition of *L. casei* cell components by TLR2 is not essential for IL-12 production (Fig. 9), as has previously been reported by us.[13] However, the peptidoglycans of *L. casei* and *L. plantarum* inhibited *L. casei*-induced IL-12 production by the TLR2-deficient macrophages only by 12 and 48%, respectively, whereas these values were 61 and 97%, respectively, in the case of the wild-type macrophages. Lipopolysaccharide inhibited IL-12 production by the wild-type and TLR2-deficient macrophages. These results suggest that the TLR2-mediated recognition of peptidoglycan plays an important role

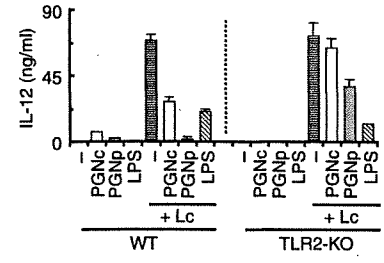


Figure 9. Toll-like receptor 2 (TLR2) is involved in the inhibition of *Lactobacillus casei*-induced interleukin-12 (IL-12) production by intact peptidoglycan. Peritoneal macrophages prepared from wild-type (WT) or TLR2-deficient (TLR2-KO) C57BL/6 mice were cultured with the intact peptidoglycans (10 μg/ml) of *L. casei* (PGNc) and *Lactobacillus plantarum* (PGNp) and lipopolysaccharide (LPS, 1 μg/ml) in the absence or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12 in culture supernatants were determined by enzyme-linked immunosorbent assay. Data are the mean ± standard deviation of triplicate cultures. Experiments were repeated twice with similar results; –, no additives or *L. casei* only.

in the inhibition of IL-12 production, and we assume that some other mechanisms may also be involved.

### Digestion products of peptidoglycan inhibit IL-12 production in a TLR2-independent manner

Watanabe *et al.*[10] reported that MDP, a product of peptidoglycan digestion, was recognized by the NOD2 receptor, and subsequently acted as a negative regulator of IL-12 production. Therefore, the effects of MDP and L18-MDP on IL-12 production were examined. L18-MDP is a stearoyl fatty acid derivative of MDP and is more effectively internalized into cells. We observed that L18-MDP but not MDP inhibited *L. casei*-induced IL-12 production, although the inhibitory effect was slightly weaker than that of *L. casei* peptidoglycan (Fig. 10a). C12-DAP, a derivative of γ-D-glutamyl-meso-diaminopimelic acid, is a ligand for NOD1, and it did not inhibit IL-12 production. The inhibitory effect of L18-MDP on IL-12 production was observed in TLR2-deficient macrophages as well as wild-type macrophages (Fig. 10b), suggesting that the digestion products of peptidoglycan can inhibit IL-12 production in a TLR2-independent manner, probably through NOD2 receptor recognition.

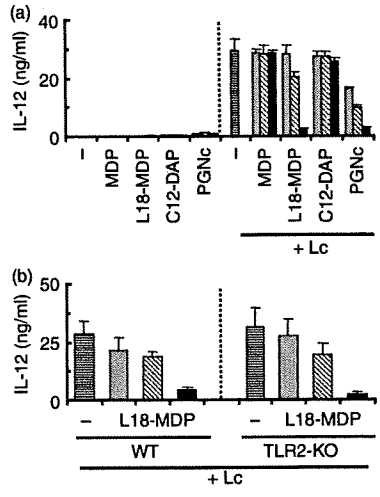


Figure 10. Toll-like receptor 2 (TLR2)-independent inhibition of interleukin-12 (IL-12) production by muramil dipeptide (MDP) derivative. (a) Peritoneal macrophages were cultured with MDP, 6-*O*-stearoyl-MDP (L18-MDP), lauroyl-γ-D-glutamyl-meso-diaminopimelic acid (C12-DAP), and the intact peptidoglycan of *Lactobacillus casei* (PGNc) at concentrations of 3 (grey), 10 (hatched), and 30 (black) μg/ml in the absence (left) or presence (+ Lc) of *L. casei* (10 μg/ml) for 24 hr; –, no additives or *L. casei* only. (b) Peritoneal macrophages prepared from wild-type (WT) or TLR2-deficient (TLR2-KO) C57BL/6 mice were cultured with L18-MDP at concentrations of 3 (grey), 10 (hatched) and 30 (black) μg/ml in the presence of *L. casei* (10 μg/ml) for 24 hr. The levels of IL-12 in culture supernatants were determined by enzyme-linked immunosorbent assay. Data are the mean ± standard deviation for triplicate cultures. Experiments were repeated twice with similar results; –, *L. casei* only.

## Discussion

We previously reported that the ICW structure of certain *Lactobacillus* strains is essential for inducing IL-12 production by macrophages, and *Lactobacillus* strains that have a rigid cell wall are resistant to intracellular digestion by macrophages and can effectively stimulate them to secrete IL-12.[13] The results of the present study revealed that, when two types of lactobacilli (sensitive to intracellular digestion by macrophages and resistant to intracellular digestion) are used to simultaneously stimulate macrophages, the sensitive *Lactobacillus* strains inhibit the IL-12 production induced potently by the resistant strains. The findings demonstrate that the sensitive strains not only rapidly lose the cell wall structure responsible for their ability to induce IL-12 production but also have the ability to actively inhibit IL-12 production. Further, our results revealed that the peptidoglycan from lactobacilli was responsible for the inhibition of IL-12 production.

Peptidoglycan is a characteristic cell wall component of Gram-positive bacteria and can stimulate macrophages and dendritic cells to secrete proinflammatory cytokines.[17,18] In our study, we found that peptidoglycan potently induced TNF-α production in macrophages at similar levels to those induced by *L. casei*. In addition, it induced IL-12 production, albeit weakly, and also induced the expression of IL-12p35 mRNA more potently than did *L. casei* (Figs 6b and 7a). However, peptidoglycan could inhibit the IL-12p40 mRNA expression potently induced by the resistant *Lactobacillus* strains, thereby inhibiting bioactive IL-12p70 secretion. This novel observation suggests that peptidoglycan can act in both pro-inflammatory and anti-inflammatory ways. Dysregulated overproduction of IL-12 and the subsequent overactivation of T helper (Th)1 cells are considered to be one of the causes of autoimmune diseases and inflammatory bowel diseases.[6,19] Recently, it has been reported that IL-17-producing Th17 cells also play critical roles in the development of inflammation in such diseases.[7,20] IL-12p40 is a subunit of IL-23, which is an important cytokine for the proliferation and maintenance of Th17 cells. Therefore, peptidoglycan may aid in the elimination of these inflammatory diseases by suppressing the production of both IL-12 and IL-23. Further studies are required to assess the possible anti-inflammatory activity of peptidoglycan.

The cell walls of *L. casei* as well as *L. johnsonii* are composed of peptidoglycan and associated uncharged polysaccharides, whereas the cell wall of *L. plantarum* is composed of peptidoglycan and associated anionic



 

polysaccharides, i.e. wall teichoic acids.[21] Although the ICW of *L. casei* was resistant to *N*-acetylmuramidase treatment, polysaccharide-depleted ICW, i.e. intact peptidoglycan, was sensitive to the enzyme treatment. The ICWs of *L. johnsonii* and *L. plantarum* were sensitive to the enzyme treatment, and their peptidoglycans were more sensitive than the corresponding ICWs (Fig. 4). These results suggest that the peptidoglycan-associated polysaccharide moiety plays a role in protecting peptidoglycan from enzyme digestion by sterically hindering access of the enzyme to the corresponding digestion sites in peptidoglycan.[22,23] Furthermore, the ICWs of these three strains showed different abilities to induce cytokines in macrophages and to inhibit *L. casei*-induced IL-12 production (Fig. 4), while the peptidoglycans of all these strains similarly induced very low levels of IL-12 and inhibited *L. casei*-induced IL-12 production (Fig. 6). The findings suggest that differences in the characteristics of the peptidoglycan-associated polysaccharide moiety, such as chemical composition, physical length and density, and electrostatic properties, are important factors determining the abilities of *Lactobacillus* strains to regulate cytokine production.

While the peptidoglycans of these three strains and the ICWs of *L. johnsonii* and *L. plantarum* were sensitive to intracellular digestion and inhibited IL-12 production, the ICW of *L. casei* was resistant to intracellular digestion and did not inhibit IL-12 production. On the basis of these results, we proposed the following hypothesis: the products of peptidoglycan digestion, such as MDP, are responsible for the inhibitory activity of peptidoglycan. Unexpectedly, the inhibitory activity of peptidoglycan was very low in TLR2-deficient macrophages, although TLR2 cannot recognize digests of peptidoglycan;[24] this suggests that the recognition of undigested peptidoglycan by TLR2 plays an important role in the inhibition of IL-12 production. This apparent contradiction may be explained by the possibility that the accessibility of TLR2 to peptidoglycan was merely correlated to the accessibility of cell wall-digesting enzymes to peptidoglycan, and that the important component for the inhibition of IL-12 production is actually undigested peptidoglycan and not the products of peptidoglycan digestion. In other words, although the recognition of peptidoglycan by TLR2 might be sterically hindered by the polysaccharide moiety in the case of the ICW of *L. casei*, the absence of the polysaccharide moiety might allow TLR2 to recognize peptidoglycan and simultaneously change the sensitivity of the *L. casei* ICW to enzymatic digestion.

Recently, Travassos *et al.*[25] reported that highly purified peptidoglycan dose not seem to signal via TLR2. They suggested that the TLR2 stimulatory activity of crude peptidoglycan preparations obtained from Gram-positive bacteria is likely to be mediated by contaminating LTA. In contrast, Dziarski and Gupta[24] re-evaluated activation of TLR2 by peptidoglycan using highly purified peptidoglycan, and showed that peptidoglycan actually stimulates TLR2. In the present study, we obtained highly purified intact peptidoglycan with no detectable contaminating LTA by treatment of heat-killed lactobacilli with detergent, organic sorbent, nuclease, pronase, and hydrogen fluoride, which is similar to the peptidoglycan purification method of Travassos *et al.*[25] The TLR2-mediated ability of the intact peptidoglycan to inhibit *L. casei*-induced IL-12 production was abolished by treatment with *N*-acetylmuramidase, which digests peptidoglycan but not possible traces of LTA and other contaminants in the peptidoglycan preparation (Fig. 6a). The results support the idea that the intact peptidoglycan of lactobacilli actually signals via TLR2 to inhibit IL-12 production, although they do not completely exclude the possibility that contaminants in the peptidoglycan preparation may be responsible for the inhibitory effect on IL-12 production.

Although the recognition of peptidoglycan by TLR2 was essential for the inhibition of IL-12 production, 12–48% of the IL-12 production in TLR2-deficient macrophages was inhibited by peptidoglycan, suggesting that other TLR2-independent mechanisms might also be involved. Watanabe *et al.*[10] reported that MDP inhibited IL-12 production after it was recognized by the intracellular receptor NOD2. We also observed that L18-MDP, a derivative of MDP, could inhibit IL-12 production. MDP might not be inhibitory because of the low efficacy of its internalization into peritoneal macrophages, which we used in our study. Collectively, these results suggest that peptidoglycan can inhibit IL-12 production by the recognition of both its undigested form via TLR2 and its digested form (MDP) via NOD2. We obtained the preliminary finding that peptidoglycan enhanced the mRNA expression of TLR2 and NOD2 in macrophages (data not shown). The regulation of these receptors by their ligand will be an interesting issue to be examined.

The inhibition of IL-12 production by peptidoglycan was mediated by neither the inhibition of phagocytosis nor the induction of the suppressive cytokine IL-10. Although we showed that IL-12 production was inhibited as a result of the reduction in IL-12p40 mRNA expression, the details of the underlying mechanisms remain to be clarified. Recently, Kuwata *et al.*[26] demonstrated that the nuclear transcription factor IκBNS inhibits IL-12p40 mRNA expression, and it was reported to be highly expressed in the macrophages of the colonic lamina propria, which hardly produced any IL-12p40 in response to bacterial components.[27] We observed strong expression of IκBNS mRNA in peritoneal macrophages after stimulation with the peptidoglycan of *L. casei*; however, this was not observed after stimulation with its ICW (K. Shida, J. Kiyoshima-Shibata and M. Nanno, unpublished data). Peptidoglycan might suppress IL-12 production by inducing such inhibitory transcription factors.