Vaccine 26 (2008) 6027–6030

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Efficient induction of oral tolerance by fusing cholera toxin B subunit with allergen-specific T-cell epitopes accumulated in rice seed

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ARTICLE INFO

Article history:
Received 30 June 2008
Received in revised form 5 September 2008
Accepted 6 September 2008
Available online 24 September 2008

Keywords:
Cholera toxin B subunit
Oral tolerance
Rice seed

ABSTRACT

Cholera toxin B (CTB) subunit is an efficient mucosal carrier molecule for induction of oral tolerance to antigens and allergens. Here, T-cell epitopes of Cry j 1 and Cry j 2, major allergens in Japanese cedar pollen, were expressed in rice seed as a fusion protein with either CTB or rice glutelin as a control. Feeding mice with rice seed containing CTB-fused T-cell epitopes suppressed allergen-specific IgE responses and pollen-induced clinical symptoms at 50-fold lower doses of T-cell epitopes than required when using control seed. Our findings present a novel potential strategy for immunotherapy of type-I allergy.

1. Introduction

Oral peptide immunotherapy using dominant T-cell epitopes provides a safe, easy, and effective strategy against allergic and autoimmune diseases [1,2]. We have recently reported that feeding mice with transgenic rice seed containing T-cell epitopes derived from major Japanese cedar pollen allergens Cry j 1 and Cry j 2 suppressed pollen-induced allergic responses and clinical symptoms [3]. These results demonstrate the clinical potential of T-cell epitopes accumulated in rice seed for immunotherapy of allergic disorders.

Cholera toxin B (CTB), a GM1 ganglioside-binding subunit of cholera toxin, has been used as an efficient mucosal carrier for induction of oral tolerance [4–6]. Compared to native antigens/allergens, 15–800-fold lower amounts of CTB-fused antigens/allergens are sufficient for induction of tolerance, indicating promising applications of CTB for immunotherapy of allergic and autoimmune diseases [4–6]. Thus, in the present study, we expressed T-cell epitopes of Cry j 1 and Cry j 2 as a fusion protein with CTB in rice seed, and examined the efficacy of CTB-fused T-cell epitopes in our experimental mouse model of allergy.

In BALB/c mice, two dominant T-cell epitopes (P1-277–290 of Cry j 1 and P2-246–259 of Cry j 2) and one subdominant T-cell epitope (P2-70–83 of Cry j 2) have been identified [7]. These three T-cell epitopes were linked together into one peptide [7], designated 3Crp, and were expressed in rice seed as a fusion protein with either CTB (CTB-3Crp) or rice glutelin acidic subunit (GLU-3Crp) as a control. In feeding experiments, a relatively high dose of GLU-3Crp control seed (equivalent to 15 μg of 3Crp) was required for suppression of allergen-specific IgE responses, whereas a much lower dose of CTB-3Crp seed (equivalent to 0.3 μg of 3Crp) was sufficient. These results indicate that the dose of 3Crp required for oral tolerance induction was decreased approximately 50-fold by fusing with CTB.

2. Materials and methods

2.1. Plasmid construction and rice transformation

A DNA coding for 3Crp was designed using optimized codons frequently used by rice seed storage protein genes [8–10]. First, two pairs of complementary DNAs coding for N- and C-terminal half regions of 3Crp were cloned into pUC19, forming a complete DNA sequence of 3Crp. This 3Crp gene was amplified by PCR, and fused to the 3′ end of the CTB gene [10], generating the expression

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doi:10.1016/j.vaccine.2008.09.019

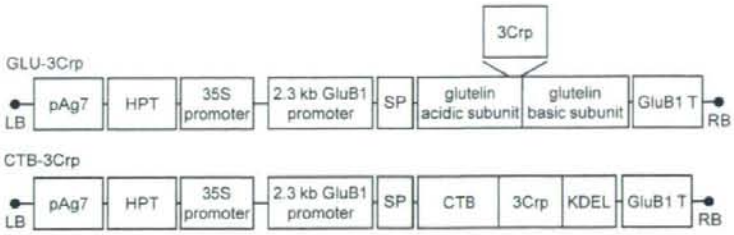

Fig. 1. Constructs for expression of GLU-3Crp and CTB-3Crp in rice seed. DNA fragments coding for GLU-3Crp and CTB-3Crp were linked to the rice seed storage protein glutelin promoter. The *hpt* gene was used for the selection of transgenic rice plants. *GluB-1*, rice glutelin *GluB-1*; 35S, cauliflower mosaic virus 35S promoter; *hpt*, hygromycin phosphotransferase gene; pAg7, agropine synthase polyadenylation signal sequence; RB, right border; LB, left border.

plasmid CTB-3Crp (Fig. 1). A KDEL endoplasmic reticulum retention signal was attached to the C-terminus of CTB-3Crp to increase accumulation levels [9]. In the control, the PCR-amplified 3Crp gene was inserted into the variable region of the C-terminal region of the glutelin GluA-2 (GLU) acidic subunit, as described [11], producing the expression plasmid GLU-3Crp (Fig. 1). These genes were ligated downstream of the 2.3-kb *GluB-1* promoter with or without a signal peptide sequence. After these two expression cassettes were separately inserted into the binary vector pGPTV-35S-HPT, they were introduced into the rice genome (*Oryza sativa* L. cv Kitaake) by *Agrobacterium tumefacience*-mediated transformation [12].

2.2. Detection of CTB-3Crp and GLU-3Crp

More than 30 independent transgenic lines were evaluated for accumulation of 3Crp in mature seed grain by Western blot analysis. Seed protein was analyzed as previously described [3]. Briefly, rice seed was ground to a fine powder, and total seed protein was extracted with extraction buffer containing 4% (w/v) SDS, 8 M urea, and 5% (w/v) β-mercaptoethanol [3]. Total seed protein was separated by electrophoresis on 15% SDS-PAGE, and transferred to Hybond-P PVDF membranes (Amersham Biosciences) for Western blot analysis. Each membrane was probed with rabbit polyclonal antibodies raised against either CTB [10] or a synthetic peptide (CKTSSSHFTFKVD: residues 23–35 of 3Crp) (Scrum, Japan), and incubated with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Promega). For quantification of the products, GLU-3Crp and CTB-3Crp proteins were expressed in *E. coli* as 6×His-tagged proteins, purified by affinity chromatography, and used as standards. Accumulation levels of 3Crp in seed were extrapolated against standard curves generated by densitometric scanning of Western blots of purified standards.

2.3. Oral tolerance induction and allergen challenge

BALB/c male mice at 6 weeks of age were purchased from CLEA Japan. Mice in groups of four to five were given 200 mg fine powder of rice seed, containing different doses of 3Crp as described in Fig. 3, orally in 1.0 ml PBS once a day for 10 days. Each dose of 3Crp in 200 mg seed powder was prepared by mixing wild-type rice seed with GLU-3Crp or CTB-3Crp seed. Mice were then intraperitoneally challenged twice on days 10 and 17 with 0.1 mg of total protein extract of Japanese cedar pollen (Cosmo Bio, Japan) adsorbed on 5 mg aluminum hydroxide (Cosmo Bio). At the first challenge, recombinant mouse IL-4 (R&D Systems) was mixed with the allergen solution at 0.1 μg per mouse to maximize the induction of allergen-specific IgE responses [3].

2.4. ELISA

On day 31, mice were bled to measure allergen-specific IgE titers by ELISA as described [3]. Briefly, ELISA plates were coated with 2 μg/ml of anti-mouse IgE (Southern Biotechnology), and serial dilutions of serum were applied to the plates. After washing, biotinylated pollen extract was used as the detection reagent. Streptavidin–horseradish peroxidase conjugate (Pierce) was then added to the plates, and the reaction was developed with peroxidase substrate solution (Moss). The last serum dilution yielding an OD 450 value of 0.1 over the background was recorded as the endpoint titer.

2.5. Histamine release and nasal symptoms

Histamine release and nasal symptoms were evaluated as described [3]. Briefly, mice in groups of 10–11 were fed wild-type rice seed, GLU-3Crp seed containing 30 μg 3Crp/grain, or CTB-3Crp seed containing 0.5 μg 3Crp/grain once a day for 10 days. These mice were intraperitoneally sensitized with total pollen extract, and then intranasally challenged with 20 μl of 1 μg/ml total pollen extract once a day for 1 week. Nasal symptoms were evaluated by counting the number of sneezes for 5 min after the last intranasal challenge. Statistical significance was determined by Student's *t*-test.

3. Results

3.1. Expression of GLU-3Crp and CTB-3Crp in rice seed

GLU-3Crp and CTB-3Crp were specifically expressed in endosperm of rice seed under the control of the 2.3-kb *GluB-1* promoter (Fig. 1). As a control for CTB-fused 3Crp, we first attempted to express 3Crp directly, but its accumulation was undetectable in the transgenic rice seed (data not shown). As an alternative, 3Crp was expressed as a fusion protein with rice glutelin, because it was known that several peptides fused with glutelin accumulated in rice seed [8,11,13]. In rice endosperm, endogenous glutelin is first synthesized as a precursor, and then post-translationally processed into two mature acidic and basic subunits [11]. As shown in Fig. 2, a GLU-3Crp signal was detected as a part of the acidic subunit with the expected molecular mass of the mature form of GLU-3Crp (34 kDa) at 35 μg/grain, whereas the precursor form of GLU-3Crp (56 kDa) was not detected. On the other hand, CTB-3Crp was detected as a band with the expected molecular mass of 17 kDa at 0.4 μg/grain (Fig. 2).

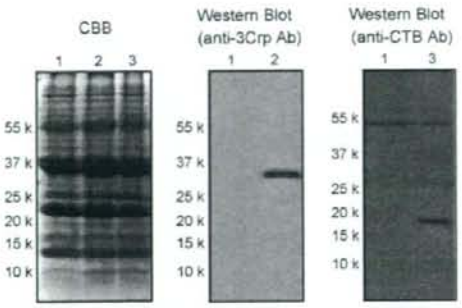

Fig. 2. Detection of GLU-3Crp and CTB-3Crp. Total protein of rice seed was separated by electrophoresis on 15% SDS-PAGE, transferred to PVDF membranes for Western blot analysis, and probed with anti-3Crp or anti-CTB antibody. Lane 1, wild-type seed; lane 2, GLU-3Crp seed; lane 3, CTB-3Crp seed.

3.2. Suppression of allergen-specific IgE responses by feeding of CTB-3Crp seed

Next, we examined the effect of orally administered rice seed containing GLU-3Crp (GLU-3Crp seed) or CTB-3Crp (CTB-3Crp seed) on pollen allergen-specific IgE responses. In our experimental mouse model, oral administration of a relatively high dose of GLU-3Crp seed (equivalent to more than 15 μg of 3Crp) were required for suppression of allergen-specific IgE, whereas lower amounts of GLU-3Crp seed (equivalent to 0–6 μg of 3Crp) did not affect IgE levels (Fig. 3). In the groups of mice orally administered with CTB-3Crp seed containing 0.3, 0.5, and 5 μg of 3Crp, the development of allergen-specific IgE was inhibited (Fig. 3). These results indicate that the dose of 3Crp required for suppression of allergen-specific IgE responses was decreased approximately 50-fold by fusing 3Crp with CTB.

3.3. Suppression of pollen-induced histamine release and clinical symptoms by feeding of CTB-3Crp seed

Levels of histamine release were 52.4 ± 12.7 ng/ml in the group of mice fed wild-type rice seed [3], whereas oral feeding of GLU-3Crp seed (containing 30 μg 3Crp) and CTB-3Crp seed (containing 0.5 μg 3Crp) suppressed levels of histamine release to 20.5 ± 10.0 and 7.5 ± 3.1 ng/ml, respectively ($P = 0.076$). In the control group of mice fed wild-type rice seed, significant nasal symptoms of sneezing developed (Fig. 4). In contrast, the number of sneezes was reduced in the group of mice fed GLU-3Crp seed (containing 30 μg 3Crp) and CTB-3Crp seed (containing 0.5 μg 3Crp) (Fig. 4). These results indicate that feeding of CTB-3Crp seed was also 60-fold more effective than GLU-3Crp seed at inhibiting allergic symptoms in the nasal tract.

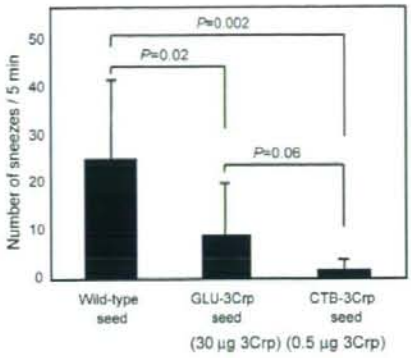

Fig. 4. Suppression of pollen-induced clinical symptoms. Mice in groups of 10–11 were fed rice seed, intraperitoneally sensitized with total pollen extract, and intranasally challenged with total pollen extract once a day for 1 week. Nasal symptoms were evaluated by counting the number of sneezes for 5 min after the last intranasal challenge.

4. Discussion

Allergen-specific IgE antibodies play a major biological role in the induction of inflammatory responses; therefore suppression of allergen-specific IgE is expected to be a key strategy for modulating allergic disorders [14]. In the present study, we showed that threshold amounts of 3Crp required for suppression of allergen-specific IgE responses were dramatically decreased by feeding of CTB-3Crp seed. Further, we demonstrated that allergic symptoms were suppressed in the group of mice fed CTB-3Crp seed. Our findings indicate that the clinical efficacy of 3Crp was significantly enhanced by fusing 3Crp with CTB.

The efficacy of CTB-fused antigens for modulating Th1-mediated autoimmune responses is well established [5]. Concerning Th2-driven responses, effects of CTB fusion were recently examined using OVA as a model antigen, showing that CTB-fused

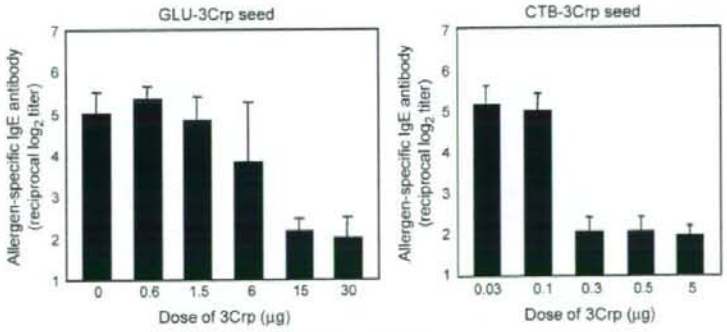

Fig. 3. Suppression of allergen-specific IgE responses. Mice in groups of four to five were orally administered once a day for 10 days with finely powdered rice seed containing different doses of 3Crp. Mice were then intraperitoneally challenged with total protein extract of Japanese cedar pollen, and allergen-specific serum IgE titers were determined by ELISA.

OVA induces oral tolerance at a 100-fold lower dose than control OVA [6,15]. Our results using pollen allergen-derived T-cell epitopes are consistent with these reports, providing additional evidence supporting the potential of CTB fusion to modulate Th2-mediated immune responses.

Based on high-affinity binding to GM1 ganglioside, CTB is considered to act as an efficient mucosal carrier molecule for induction of oral tolerance. However, CTB has recently been shown to have immunomodulatory properties, which are associated with induction of oral tolerance. A recent study showed that oral administration of CTB-fused OVA induces regulatory T-cells with strong suppressive activities [15]. Another study reported that intranasal administration of CTB-free Bet 1 v allergen suppressed allergen-specific IgE, IgG2a, and IgA responses, whereas CTB-fused Bet v 1 enhanced allergen-specific IgG2a and IgA levels, suggesting that CTB modifies the immunological properties of Bet 1 v [16]. Further, orally administered CTB has adjuvant activities such as induction of cellular activation, surface molecule expression, and cytokine production [17]. Although the mechanism is not clear, these immunomodulatory properties of CTB might be a clue to explain the reduction of histamine release and sneezes in CTB-3Crp-fed mice compared to GLU-3Crp-fed mice, while no significant differences in allergen-specific IgE levels were observed between the two groups (Fig. 3). Further studies on the effect of CTB are required prior to future clinical applications of CTB-fused T-cell epitopes.

Production platforms using rice seed offer several benefits such as low-cost production and storage, and safety without contamination by mammalian pathogens. Rice seed also provides convenient and effective mucosal delivery of antigens/allergens for immunotherapy [3]. Our study here showed that the efficacy of 3Crp was significantly improved by genetic fusion with CTB, further supporting the potential of rice seed containing T-cell epitopes for clinical applications in immunotherapy of type-I allergy.

Acknowledgements

This work was supported by a "Functional analysis of genes relevant to agriculturally important traits in rice genome" project grant and a "Genomics for Agricultural Innovation GMC0010" project grant from the Ministry of Agriculture, Forestry and Fisheries of Japan and a grant from the Bio-oriented Technology Research Advancement Institution (BRAIN) to F. Takaiwa, and KAKENHI (19780012) to H. Takagi.

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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-

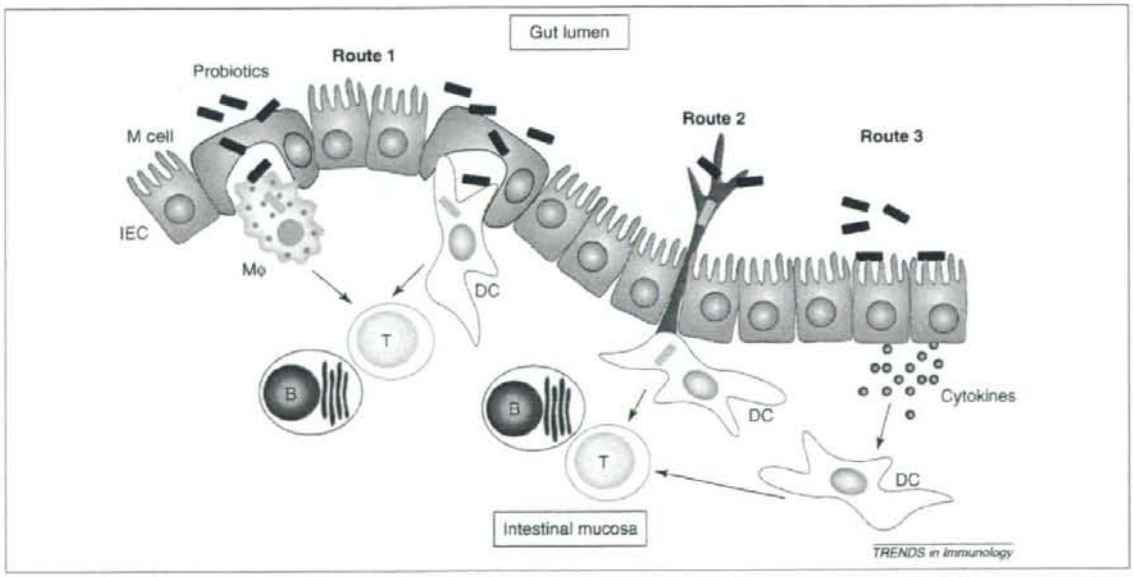

Figure 1. Three hypothetical pathways by which probiotics can trigger and modulate immune function in the intestine. **(a)** Specialized epithelial cells called M (microfold) cells in the follicle-associated epithelium covering Peyer's patches or in the villi can take up probiotics directly by transcytosis. 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

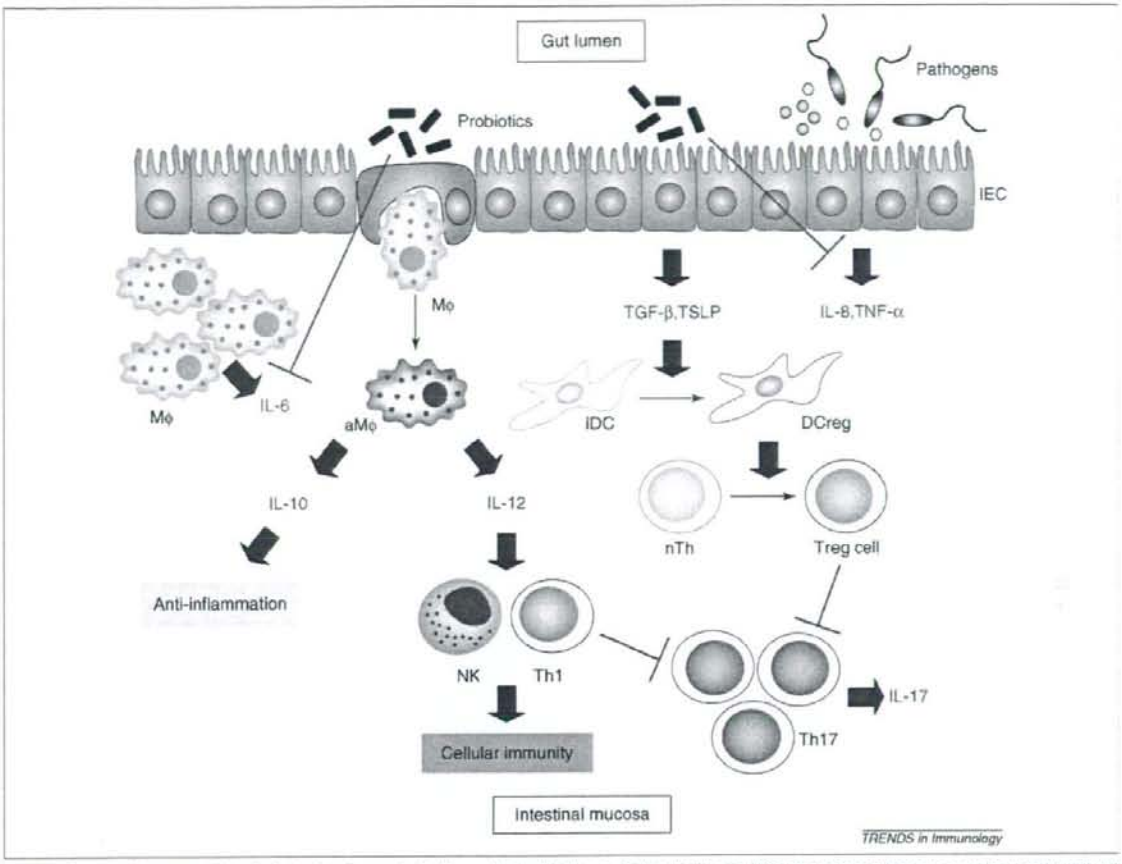

Figure 2. Supposed immune cell circuits in the intestine modulated by probiotics. While intestinal epithelial cells (IECs) exposed to pathogenic microbes or related stimuli produce proinflammatory mediators such as interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α), probiotics suppress the production 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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