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Lactobacillus casei Secreting α-MSH Induces the Therapeutic Effect on DSS-Induced Acute Colitis in Balb/c Mice

Yoon, Sun-Woo[1], Chul-Ho Lee[2], Jeong-Yoon Kim[3], Jie-Youn Kim[4], Moon-Hee Sung[4,5], and Haryoung Poo[1]*

[1]*Mucosal Immunology Laboratory, BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejon 305-600, Korea*
[2]*Disease Model Research Center, KRIBB, Daejon 305-600, Korea*
[3]*School of Bioscience and Biotechnology, Chungnam National University, Daejon 305-764, Korea*
[4]*BioLeaders Corporation, Daejeon 305-500 Korea*
[5]*Department of Bio and Nanochemistry, Kookmin University, Seoul 136-702, Korea*

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The neuropeptide α-melanocyte-stimulating hormone (α-MSH) has anti-inflammatory property by downregulating the expressions of proinflammatory cytokines. Because α-MSH elicits the anti-inflammatory effect in various inflammatory disease models, we examined the therapeutic effect of oral administration of recombinant *Lactobacillus casei*, which secretes α-MSH (*L. casei*-α-MSH), on dextran sulfate sodium (DSS)-induced colitis in Balb/c mice. Thus, we constructed the α-MSH-secreting *Lactobacillus casei* by the basic plasmid, pLUAT-ss, which was composed of a PldhUTLS promoter and α-amylase signal sequence from *Streptococcus bovis* strain. Acute colitis was induced by oral administration of 5% DSS in drinking water for 7 days. To investigate the effect of *L. casei*-α-MSH on the colitis, *L. casei* or *L. casei*-α-MSH was orally administered for 7 days and their effects on body weight, mortality rate, cytokine production, and tissue myeloperoxidase (MPO) activity were observed. Administration of *L. casei*-α-MSH reduced the symptom of acute colitis as assessed by body weight loss (DSS alone: 14.45±0. 2 g; *L. casei*-α-MSH: 18.2±0.12 g), colitis score (DSS alone: 3.6±0.4; *L. casei*-α-MSH: 1.4±0.6), MPO activity (DSS alone: 42.7±4.5 U/g; *L. casei*-α-MSH: 10.25±0.5 U/g), survival rate, and histological damage compared with the DSS alone mice. *L. casei*-α-MSH-administered entire colon showed reduced *in vitro* production of proinflammatory cytokines and NF-κB activation. The α-MSH-secreting recombinant *L. casei* showed significant anti-inflammatory effects in the murine model of acute colitis and suggests a potential therapeutic role for this agent in clinical inflammatory bowel diseases.

*Corresponding author
Phone: 82-42-860-4157; Fax: 82-42-860-4593;
E-mail: haryoung@mail.kribb.re.kr

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Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a chronic inflammatory disorder of the intestinal tract and its symptoms are diarrhea, weight loss, and abdominal pain [29]. This inflammatory disorder results from altered mucosal immune function, involving the mucosa in the colon. For example, activated immune cells, such as neutrophils, macrophages, and cytotoxic T cells, play the role of aggressors that destroy the intestinal barrier either directly through physical contact or indirectly through the release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [28, 29]. In the IBD murine model, histological analysis typically reveals extensive crypt and epithelial cell damage, significant infiltration of granulocytes and mononuclear immune cells, tissue edema, and often frank ulceration [26, 38]. The oral administration of dextran sulfate sodium (DSS), a heparin-like polysaccharide, induces weight loss, loose stool/diarrhea, and occult and gross rectal bleeding in mice. The DSS model has been useful for examining the fundamental mechanisms underlying the inflammatory pathophysiology associated with IBD [18, 39].

The α-melanocyte-stimulating hormone (α-MSH), a 13-amino-acid neuropeptide produced by intracellular cleavage of the pro-opiomelanocortin (POMC) hormone, mediates communication between the nervous and immune systems [30]. α-MSH has been shown to exert its cellular effects by binding to five different G protein-coupled receptors called melanocortin receptors (MC_1R through MC_5R) [13]. Previous studies demonstrated that α-MSH

exhibits anti-inflammatory activity in various disease models, including arthritis, inflammatory bowel disease, and endotoxemia/ischemia, suggesting that α-MSH may be a promising candidate therapeutic drug for inflammatory diseases [6, 7, 21, 31]. In addition, α-MSH antagonizes the effects of proinflammatory cytokines such as IL-1β, IL-6, interleukin-8 (IL-8), interferon-γ (IFN-γ), and TNF-α, and inhibits proinflammatory signaling events, such as activation of nuclear factor κB (NF-κB) [7, 21]. Moreover, α-MSH promotes interleukin-10 (IL-10) production, immunosuppressive cytokine, in monocytes. Human monocytes stimulated with α-MSH through its binding to MC_1R increased production of both IL-10 protein and mRNA [3]. Several recent studies showed that α-MSH treatment reduced IBD characteristics such as blood in the stool, and body weight loss in a murine IBD model [27, 31]. These observations in preclinical models of intestinal disease led to the evaluation of α-MSHs and their receptors in patients with celiac disease, where immunoreactivity for α-MSH, MC_1R, and MC_5R was detected [8]. Moreover, MC_1R gene frameshift mutation is significantly higher in DSS-induced acute colitis then in wild-type mice [22].

It has been reported that probiotics, living nonpathogenic organisms, confer health benefits to the treatment of IBD [10, 34]. *Lactobacilli* also have an advantage as a vehicle to deliver drug to mucosa, because of persistence in the gastrointestinal tract [33], and its immunomodulatory capacity may be important for the development of anti-IBD therapy. In particular, *Lactobacillus* species have been shown to decrease the secretion of proinflammatory cytokines such as TNF-α and IL-6, and increase the immunomodulatory cytokines such as IL-10 from the inflamed ileum in the colitis mice model [10, 32, 36].

Based on these findings, we constructed a *L. casei* secreting α-MSH and assessed the anti-inflammatory properties of *L. casei*-α-MSH in the DSS-induced murine colitis model by examining the intensity of the inflammatory response and disease severity after oral administration of *L. casei*-α-MSH. We found that oral administration of mice with *L. casei*-α-MSH protected acute colitis characteristic signs. These findings open an exciting new avenue for the possibility of treating intestinal diseases.

Materials and Methods

Construction of *L. casei*-α-MSH

pLUAT-ssMSH, an expression vector for secretion of α-MSH, was constructed as follows. First, the basic plasmid (pLUAT-ss) for peptide secretion in *L. casei* BLS was constructed by inserting an ldhUTLS promoter [25] and N-terminal 42 amino acids (coding a putative secretion signal) of the α-amylase gene derived from *Streptococcus bovis* (*S. bovis*) 148 [9] into pHAT:pgsA-SA [19]. The ldhUTLS promoter fragment was then amplified from pL3U with primers (5'-ACT AGT CAA AAT TAT GAA-3' and 5'-CAT ATG GTC TTT TCC TCC-3'), and a secretion signal fragment was amplified from *S. bovis* 148 with primers (5'-CAT ATG GAT CTG ATG ACA-3' and 5'-GGT ACC GGA TCC TGC ATT AAC GAC-3'). The pHAT:pgsA-SA [19] plasmid fragment, ldhUTLS promoter fragment, and secretion signal fragment were digested with SpeI-BamHI, SpeI-NdeI, and NdeI-BamHI, respectively, and these three fragments were then ligated. The resulting plasmid was designated pLUAT-ss. The α-MSH fragment was obtained by annealing with primers (5'-GGA TCC TCA TAT TCA ATG GAA CAT TTT CGT TGG GGT AAA CCA GTT TAA GGT ACC-3' and 5-C TTA AAC TGG TTT ACC CCA ACG AAA ATG TTC CAT TGA ATA TGA G-3) and inserted into pLUAT-ss digested with BamHI and KpnI. The resulting plasmid was designated pLUAT-ssMSH. The plasmid was first established in *E. coli* JM83 and transformed into *L. casei* BLS by electroporation [25]. Recombinant and expression host *L. casei* BLS cells were grown in MRS medium with erythromycin (16 μg/ml) at 30°C, and *E. coli* JM83 was grown in Luria-Bertani medium with erythromycin (300 μg/ml) at 37°C. Secretion of α-MSH in *L. casei* BLS cell culture supernatants was measured by ELISA kits according to the manufacturer's instructions (Phoenix Pharmaceuticals Inc., CA, U.S.A.).

Oral Administration of *L. casei*

Six-week-old Balb/c female mice were purchased from Koatech (Pyeongtaek, Korea) and housed in the specific pathogen-free (SPF) animal facility at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). For induction of acute colitis, mice were fed 5% DSS (ICN Biomedical, Eschwege, Germany), dissolved in water that was filter-purified, for 7 days (days 0–6 *ad libitum*) [26]. Wild-type mice received the filtered water alone. Daily changes in body weight and clinical signs of colitis, such as diarrhea and bloody stool, were assessed. At day 7 after DSS treatment, mice were daily orally administered *L. casei*, *L. casei*-α-MSH (1×10^{10}/mouse/day), or phosphate-buffered saline (PBS). After administration, mice were killed, and colon tissues were collected for cytokine production and histological study.

Tissue Myeloperoxidase (MPO) Activity

MPO activity from proximal to distal colon was measured as previously described in tissue [11]. Tissue samples were weighed and homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, U.S.A.). A sample of the homogenate was centrifuged at 12,000 ×*g* for 20 min at 4°C. After centrifugation, 100 μl of the supernatant of each sample was mixed with 2,810 μl of assay buffer [(30 μl of 20 mg/ml *o*-dianisidine dihydrochloride (Sigma), and 30 μl of 20 mM hydrogen peroxide in 50 mM potassium phosphate buffer, pH 6.0)], and the mixture was incubated at 20°C for 10 min. The reaction was then terminated by the addition of 30 μl of 2% sodium azide, and absorbance was measured at 460 nm. MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 unit per minute per gram of tissue (wet weight).

Histological Study

For each animal, histological examination was performed on three samples of the distal colon; samples were fixed in 10% formalin before staining with hematoxylin and eosin (H&E). Histological scores were assessed according to the procedures previously

described [14, 17]. Briefly, the scores were graded from 0 to 4 as follows in a blinded fashion: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, thickening of the colon wall, and focal loss of crypts; and 4, transmural infiltrations, massive loss of goblet cell, and diffuse loss of crypts.

Colitis Score

Body weight, occult or gross blood lost per rectum, and stool consistency were determined every other day during the colitis induction. The colitis score was assessed by trained individuals blinded to the treatment groups [35]. The baseline colitis score was determined on day 0. Briefly, no weight loss was scored as 0, weight loss of 1% to 5% from baseline as 1; 5% to 10% as 2; 10% to 20% as 3; and more than 20% as 4. For stool consistency, a score of 0 points was assigned for well-formed pellets, 2 points for pasty and semi-formed stools that did not adhere to the anus, and 4 points for liquid stools that did adhere to the anus. For bleeding, a score of 0 point was assigned for no blood, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added together and divided by three, resulting in a total clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis).

ELISA for Cytokines

TNF-α, IL-1β, IL-4, IL-6, and IL-10 concentrations in culture supernatants of colonic segments were measured by ELISA kits according to the manufacturer's instructions (BD Bioscience, Franklin Lakes, NJ, U.S.A.). Thus, the mouse colon was removed and washed in PBS, and the colon was then cut into small pieces. Colon segments were incubated with 1.0 ml of RPMI containing 10% serum in a well of 12-well culture plates at 37°C for 24 h. The cultured medium was harvested and cytokine levels were determined.

Western Blot Analysis

The mouse entire colon was removed, washed in PBS, and homogenized in a buffer containing 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF) in PBS, pH 7.4. Samples were incubated for 30 min on ice and centrifuged (12,000 rpm, 20 min, 4°C). Protein was collected from the supernatants and the concentration determined by following Bradford's method. Equivalent amounts of protein (30 μg/lane) were size-fractionated in a 12% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS-T (TBS containing 0.05% Tween-20) and blotted with rabbit anti-IκB-α polyclonal and rabbit anti-phospho IκB-α polyclonal antibody (Cell Signaling Technology, Beverly, MA, U.S.A.). The blots were developed using an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) and visualized by chemiluminescence using an ECL kit (Amersham Biosciences, Uppsala, Sweden).

RESULTS

Construction of α-MSH Secreting *L. casei* BLS

To direct the secretion of α-MSH by *L. casei* BLS, we have constructed recombinant *L. casei* secreting biologically active α-MSH (Fig. 1A). Thus, we generated an *L. casei*-α-MSH (pAT:α-MSH) vector fused to three fragments of the PIdhUTLS promoter, α-amylase signal sequence, and α-MSH. In order to examine the level of α-MSH secreted, *L. casei* and *L. casei*-α-MSH BLS were grown overnight at 30°C and then the amount of α-MSH in the culture supernatants of *L. casei* or *L. casei*-α-MSH BLS was determined by ELISA. As shown in Fig. 1B, ELISA analysis revealed that a significant increase of α-MSH secretion was observed in *L. casei*-α-MSH BLS (270.8± 12.5 pg/ml) compared with *L. casei* (10.9±1.3 pg/ml). Next, we checked the secretion of α-MSH in colon lavage fluids. After oral administration of Balb/c mice with one

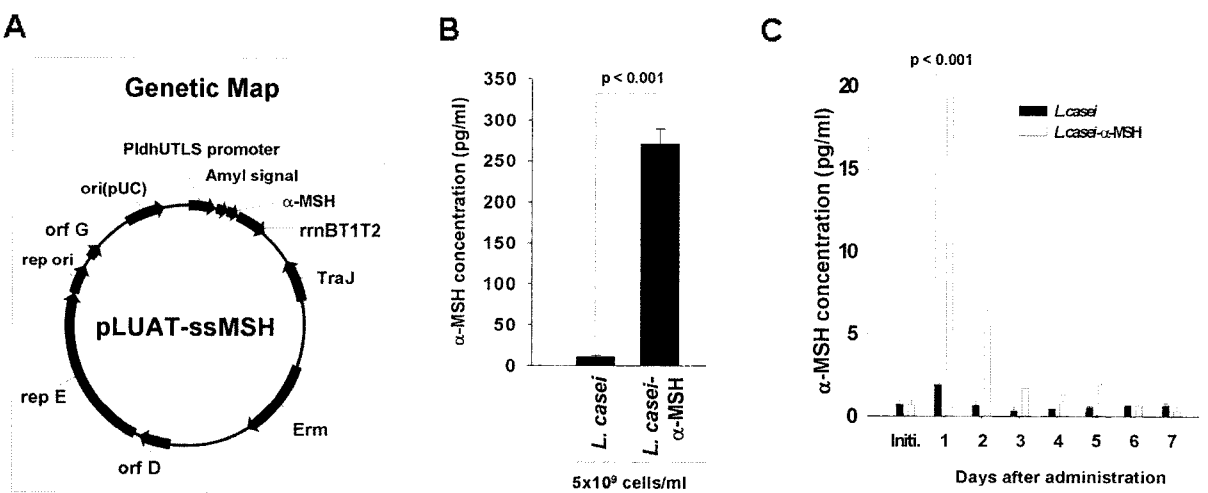

Fig. 1. Secretion of α-MSH by *L. casei*.

A. A schematic diagram of pLUAT-ssMSH plasmid for α-MSH secretion on the *L. casei* BLS. **B**. The concentration of α-MSH in *L. casei* culture supernatant as determined by ELISA. **C**. Oral administration of mice with one dose of *L. casei* or *L. casei*-α-MSH BLS cells culture supernatants. Mice were daily sacrificed, and the colon was removed and washed 4 times with 1 mM EDTA in HBSS. α-MSH concentration in colon lavage fluids was determined by ELISA. n=10 mice per group.

dose of *L. casei* or *L. casei*-α-MSH BLS cells culture supernatants, mice were sacrificed. After oral administration on day 1, α-MSH was detected in colon lavage fluids (*L. casei*: 1.9± 0.1 pg/ml; *L. casei*-α-MSH BLS: 19.3±0.4 pg/ml). However, after oral administration on day 2, significant amount of α-MSH was not detected in colon lavage fluids (Fig. 1C).

Treatment with *L. casei*-α-MSH Reduces Inflammatory Responses in DSS-Induced Acute Colitis

We next evaluated the effect of *L. casei*-α-MSH on the production of inflammatory mediators that are mechanistically linked to DSS-induced colitis. Mice were daily orally administered *L. casei* or *L. casei*-α-MSH for 7 days after acute colitis induction, and TNF-α, IL-1β, and IL-6 proinflammatory cytokine analysis was performed on the colonic segments culture supernatants by ELISA. After oral administration on day 7, TNF-α levels were significantly attenuated in the *L. casei*-α-MSH-administered group (336.75±33.9 pg/ml) compared with the DSS-alone group (980.12±43.6 pg/ml) (Fig. 2A). IL-1β levels were markedly reduced in the *L. casei*-α-MSH-administered group (166.64±13.7 pg/ml) compared with the DSS-alone group (435.9±23.8 pg/ml) (Fig. 2B). Significantly reduced levels of IL-6 were observed in the *L. casei*-α-MSH- administered group (220.6±12.7 pg/ml) compared with the DSS-alone group (665.1±42.6 pg/ml) (Fig. 2C). These results suggest that *L. casei*-α-MSH administration deactivates inflammatory response in the colonic mucosa.

Previous studies suggest that NF-κB, a crucial regulator of the immune system and the inflammatory response, is strongly activated during experimentally induced colitis [1, 12, 37]. In general, NF-κB activation is determined by examining phosphorylation of IκB, because degradation

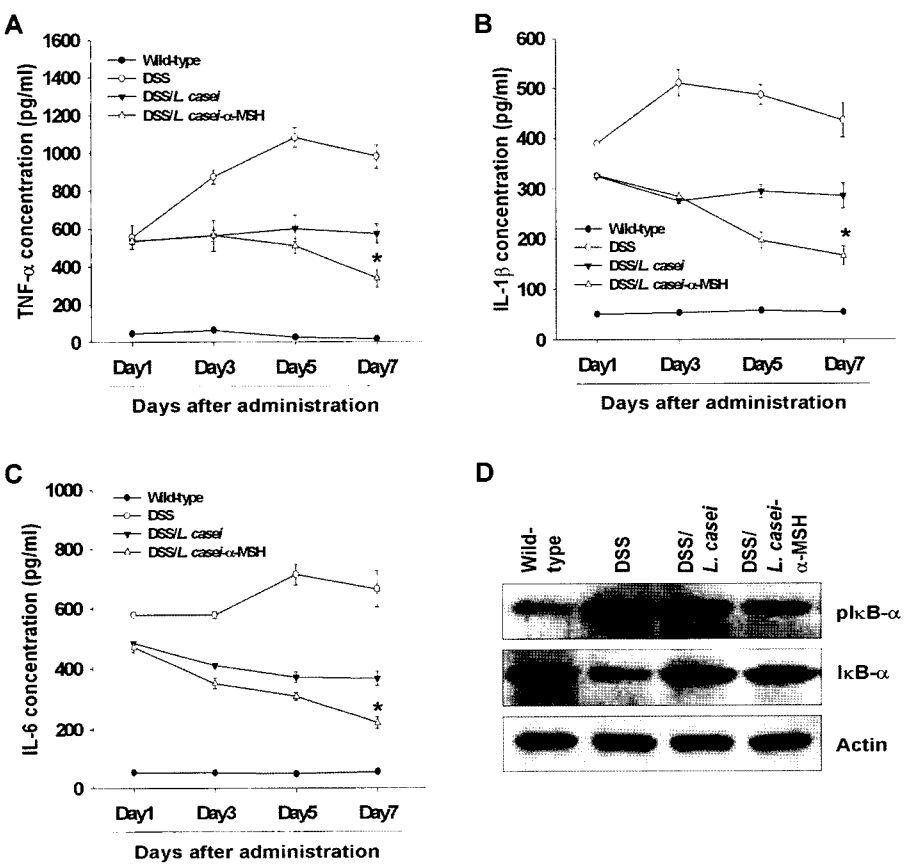

Fig. 2. Inhibitory effect of *L. casei*-α-MSH on proinflammatory cytokine production in DSS-induced acute colitis mice.
Acute colitis was induced by administration of DSS in drinking water for 7 days. After acute colitis induction, mice were daily orally administered *L. casei* or *L. casei*-α-MSH (1×10^{10}/mouse/day) for 7 days. After sacrifice, the level of cytokines was checked in the incubation medium (RPMI 1640 supplemented with 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 μg/ml concanavalin A) after 24 h of culture of colonic segment. TNF-α (**A**), IL-1β (**B**), and IL-6 (**C**) secretion were determined by ELISA. The data presented in this figure are representative of triplicate experiments. Statistical significance was assessed compared with the DSS-alone group. *$p<0.001$. Data are means ± standard errors; n=10 per group. **D**. Acute colitis was induced by administration of DSS in drinking water for 7 days. After induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH (1×10^{10}/mouse/day) for 7 days. After the final administration, the entire colon was removed from the wild-type and experimental animals, and tissue segments were then homogenized. The IκB-α, phosphorylated IκB-α (pIκB-α), and actin were determined by Western blot analysis. The data presented in this figure are representatives of triplicate experiments. n=5 mice per group.

of IκB *via* its phosphorylation is necessary for nuclear translocation of NF-κB and subsequent activation of target gene expression [16]. Therefore, we investigated whether *L. casei*-α-MSH played an important role in the DSS-induced anti-inflammatory effect by analyzing the entire colon tissue for NF-κB activation by Western blot analysis. As shown in Fig. 2D, the orally administered of *L. casei*-α-MSH group was found to have significantly reduced IκB-α activation compared with the DSS-alone group.

Treatment with *L. casei*-α-MSH Increases Immunoregulatory Cytokine Production in DSS-Induced Acute Colitis

Immunoregulatory cytokines such as IL-4 and IL-10 modulate cytokine production in ulcerative colitis and Crohn's disease, and it has been suggested that changes in their production may be associated with the pathogenesis of IBD [2, 15, 24]. Therefore, we determined the effect of treatment of DSS-induced colitis with *L. casei*-α-MSH on the production of immunoregulatory cytokines. Mice were orally administered *L. casei* or *L. casei*-α-MSH daily for 7 days after induction of acute colitis, and the amounts of IL-4 and IL-10 cytokines in the colonic segments culture supernatants were determined by ELISA. After oral administration on day 7, IL-10 levels were increased in the *L. casei*-α-MSH-administered group (475.3±10.1 pg/ml) compared with the DSS-alone group (99.85±14.7 pg/ml) (Fig. 3A). IL-10 production in the *L. casei*-α-MSH-administered group was significantly higher than in the *L. casei*-administered group. IL-4 levels were increased in the *L. casei*-α-MSH-administered group (475.3±10.1 pg/ml) compared with the DSS-alone group (204.5±4.3 pg/ml) (Fig. 3B).

Effect of Orally Administered *L. casei*-α-MSH on the Histological Features of DSS-Induced Acute Colitis

To study histological damage of the colon with respect to crypt epithelial damage and acute inflammatory cell infiltration, the colon was stained with hematoxylin and eosin (H&E). In the DSS-alone mice, the inflammatory patterns observed included focal erosion of the epithelium, crypt shortening, and various degrees of inflammatory cells infiltrating the submucosa. In contrast, when mice were administered *L. casei*-α-MSH, the inflammatory patterns were significantly reduced compared with the DSS-alone group (Fig. 4A). Consistent with the results of histological analysis, the histological score of the *L. casei*-α-MSH-administered group was lower than that of the *L. casei*-administered group or the DSS-alone group (Fig. 4B). To further assess the severity of colitis in these animals, mice were orally administered *L. casei* or *L. casei*-α-MSH daily for 7 days after acute colitis induction and then the colonic length was measured. Macroscopic examination of the colons demonstrated that the DSS-alone group showed a marked reduction in the average colonic length over that of the *L. casei*-α-MSH-administered group (Figs. 4C, and 4D)

Treatment with *L. casei*-α-MSH Protects Against DSS-Induced Acute Colitis Development

We next investigated the therapeutic efficacy of *L. casei*-α-MSH treatment in the DSS-induced colitis. Thus, we observed symptomatic parameters such as body weight loss, survival rate, MPO activity, and colitis score caused by DSS-induced acute colitis. During induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH. As shown in Fig. 5A, the *L. casei*-α-

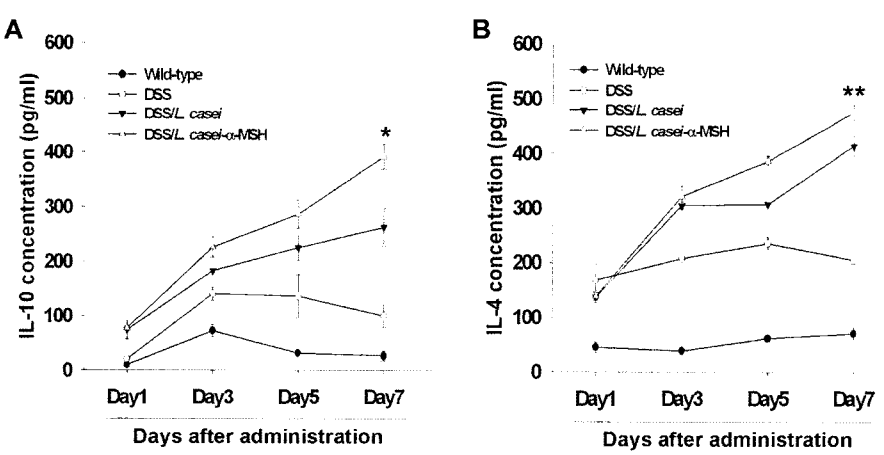

Fig. 3. *L. casei*-α-MSH enhances immunoregulatory cytokine in DSS-induced acute colitis mice.
Acute colitis was induced by administration of DSS in drinking water for 7 days. After induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH (1×10^{10}/mouse/day) for 7 days. After sacrifice, the levels of cytokines were determined in the incubation medium (RPMI 1640 supplemented with 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 μg/ml concanavalin A) after 24 h of culture of colonic segment. IL-10 (**A**) and IL-4 (**B**) secretions were checked by ELISA. The data presented in this figure are representatives of triplicate experiments. Statistical significance was assessed compared with the DSS-alone group. *$p<0.005$, **$p<0.001$. Data are means±standard errors; n=10 per group.

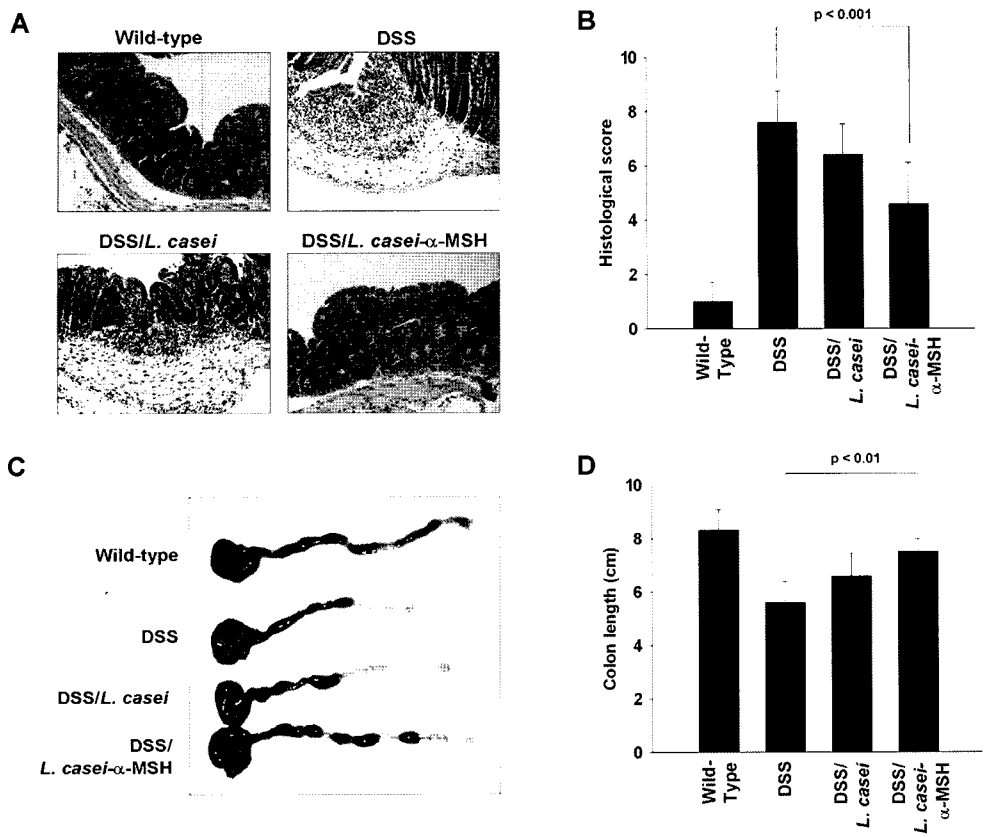

Fig. 4. Histological findings of DSS-induced acute colitis.
Acute colitis was induced by administration of DSS in drinking water for 7 days. After induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH (1×10^{10}/mouse/day) for 7 days. **A**. After sacrifice, paraffin-embedded proximal and distal segments of the colon were stained with hematoxylin and eosin (H&E). **B**. Total histological score was obtained by the severity and extent of total inflammation and crypt damage. **C**. Macroscopic findings of the colons from acute colitis mice treated with *L. casei* or *L. casei*-α-MSH. **D**. The length of the colons in each group is depicted. The data presented in this figure are representatives of triplicate experiments. A representative example of each group is shown, with n=10 mice per group.

MSH-administered group (18.2±0.12 g) showed their body weight loss protected compared with the DSS-alone group (14.45±0.2 g). However, the orally administered *L. casei*-α-MSH group showed higher protection of body weight loss in mice than orally administered mice with *L. casei*. Moreover, the *L. casei*-α-MSH-administered group had enhanced survival rate compared with the DSS-alone group. Full mortality was seen in the DSS-alone group on day 11, in the *L. casei*-administered group on day 15, and *L. casei*-α-MSH-administered group on day 19 (Fig. 5B). Additionally, we determined the level of MPO activity, and the colitis score effect of *L. casei*-α-MSH was observed in acute colitis. Mice were daily orally administered *L. casei* or *L. casei*-α-MSH for 7 days after acute colitis induction. After oral administration on day 7, the MPO activity was found to be significantly reduced in the *L. casei*-α-MSH-administered group (10.25±0.5 U/g) compared with the DSS-alone group (42.7±4.5 U/g) (Fig. 5C). Colitis scores were significantly different between the DSS-alone group (3.6±0.4) and *L. casei*-α-MSH-administered group (1.4± 0.6) (Fig. 5D). These findings collectively suggest that the orally administered *L. casei*-α-MSH elicits anti-inflammatory bowel disease effects compared with the DSS-alone group.

Discussion

Probiotics, as therapeutic agents against mucosal inflammation, have been used on human clinical diseases including IBD [24]. In experimental colitis animal models, there are several reports that probiotics could prevent the development of colitis [10, 23]. Because probiotics represent a subclass of commensals that modulate mucosal innate responses and possibly exhibit an anti-inflammatory function, they are used as drug delivery systems. For example, IL-10 secreted by *Lactobacillus lactis* reduces the severity of the DSS-induced inflammatory bowel disease model system [36].

Because α-MSH has a central role in downregulating inflammatory responses [7, 20], we engineered in the present study a new method of delivering *L. casei* for use in therapeutic intervention of IBD. Our ELISA analysis

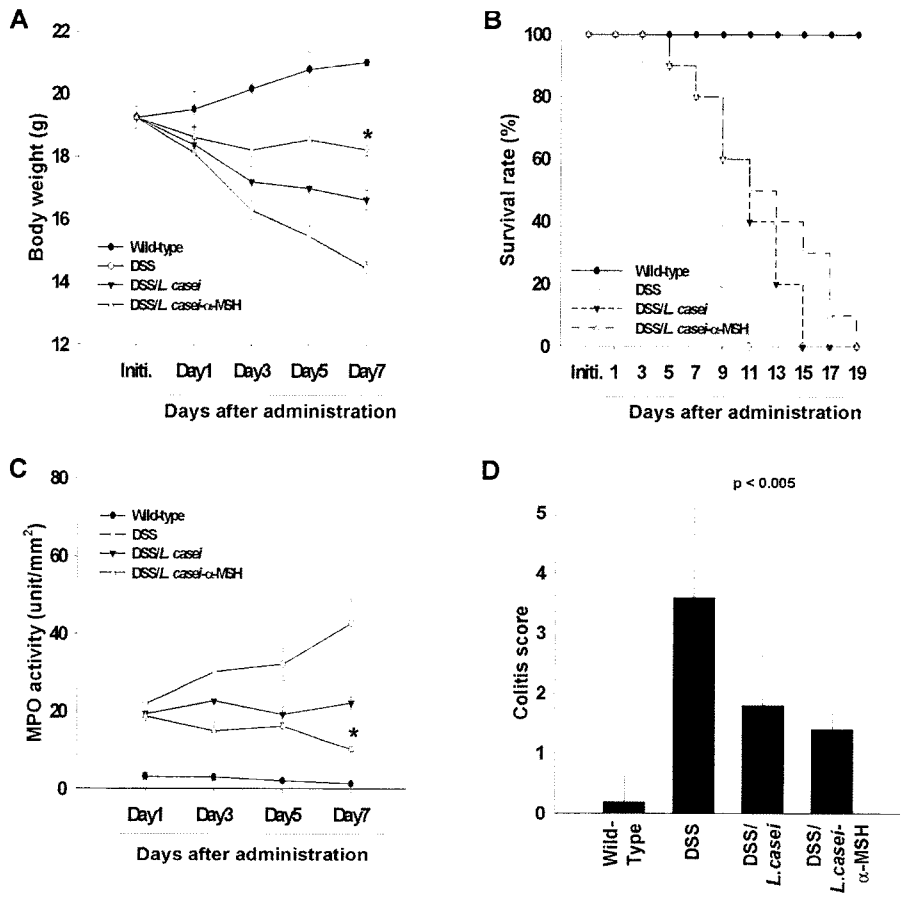

Fig. 5. Effects of *L. casei*-α-MSH on DSS-induced acute colitis mice.
During induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH (1×10^{10}/mouse/day). Clinical progression was monitored by body weight change (**A**) and survival rate (**B**). The data presented in this figure are representatives of triplicate experiments. Statistical significance was assessed compared with the DSS-alone group. *p<0.005. Data are means±standard errors; n=10 mice per group. Acute colitis was induced by administration of DSS in drinking water for 7 days. After induction of acute colitis, mice were daily orally administered *L. casei* or *L. casei*-α-MSH for 7 days. Clinical progression was monitored by tissue myeloperoxidase (MPO) activity (**C**) and colitis score (**D**) in the entire colon of different treatment groups. The data presented in this figure are representatives of triplicate experiments. Statistical significance was assessed compared with the DSS-alone group. *p<0.05. Data are means±standard errors; n=10 mice per group.

revealed that α-MSH was effectively secreted in *L. casei* cell culture supernatant in a murine experimental model of acute colitis. Daily oral administration of *L. casei*-α-MSH at the onset of the disease protected the clinical sign, abrogating body weight loss and intestinal inflammation, and reduced the full mortality rate compared with both the *L. casei*-administered group and DSS-alone group.

Infiltration of leukocytes into the mucosa has been suggested to significantly contribute to the tissue necrosis and mucosal dysfunction associated with IBD [4]. Determination of local inflammatory parameters after DSS administration revealed an effect of *L. casei*-α-MSH. Histological observation and MPO activity levels clearly showed that oral administration of mice with *L. casei*-α-MSH effectively affected inflammation compared with the *L. casei*-administered group and DSS-alone group. Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, induce the production of leukocytes, and these cytokines have been detected in mucosal biopsies of patients with IBD [29]. In particular, TNF-α is considered to be a key mediator in the pathogenesis of IBD. Other studies indicated that *L. casei* decreases the secretion of TNF-α from the inflamed ileum of Crohn's disease [5]. Likewise, our present findings showed that administration of mice with *L. casei* reduced TNF-α production in the DSS-induced acute colitis model. However, because α-MSH antagonizes the effects of TNF-α production, recombinant *Lactobacillus casei* secreting α-MSH effectively reduced the TNF-α production compared with *L. casei*-administered mice in the DSS-induced acute colitis model (Fig. 2A). Not only did this therapy result in decreased proinflammatory cytokines, but it also increased mucosal IL-10. IL-10 is an immunoregulatory cytokine, mainly produced by macrophages, dendritic cells, and T regulatory lymphocytes.

Likewise, α-MSH has been reported to induce the release of IL-10 in monocytes [3]. In our study, *L. casei*-α-MSH administration increased changes in IL-10 release by inflamed tissue compared with the *L. casei*-administered group (Fig. 4A). Thus, oral administration of *L. casei*-α-MSH significantly reduced mucosal inflammatory activity.

The NF-κB activation has biological significance in IBD, because it is highly activated in the mucosal biopsy specimens of patients with ulcerative colitis and Crohn's disease. The NF-αB antisense oligonucleotide treatment also aborts chronic intestinal inflammation in a murine model of inflammation [37]. NF-αB has been shown to activate transcription of genes encoding proinflammatory cytokines in different cell types. In our present study, *L. casei*-α-MSH administration significantly inhibited NF-αB activation in inflamed colon segments. Thus, it is possible that the underlying mechanism of the protective effects of α-MSH involves a reduction of leukocytes infiltration into the colonic mucosa, possibly *via* inhibition of TNF-α production and NF-κB activation. However, further studies are needed to confirm this possibility.

In summary, this work identified *L. casei*-α-MSH as an immunomodulatory factor with the capacity to deactivate the inflammatory response in the experimental mouse acute colitis model. Our recombinant *L. casei*, which secreted α-MSH, could be a therapeutic approach to the treatment of IBD.

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References

1. Bantel, H., C. Berg, M. Vieth, M. Stolte, W. Kruis, and K. Schulze-Osthoff. 2000. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *Am. J. Gastroenterol.* **95:** 3452–3457.
2. Barbara, G., Z. Xing, C. M. Hogaboam, J. Gauldie, and S. M. Collins. 2000. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* **46:** 344–349.
3. Bhardwaj, R. S., A. Schwarz, E. Becher, K. Mahnke, Y. Aragane, T. Schwarz, and T. A. Luger. 1996. Pro-opiomelanocortin-derived peptides induce IL-10 production in human monocytes. *J. Immunol.* **156:** 2517–2521.
4. Blumberg, R. S., L. J. Saubermann, and W. Strober. 1999. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr. Opin. Immunol.* **11:** 648–656.
5. Borruel, N., M. Carol, F. Casellas, M. Antolín, F. de Lara, E. Espín, J. Naval, F. Guarner, and J. R. Malagelada. 2002. Increased mucosal tumor necrosis factor alpha production in Crohn's disease can be downregulated *ex vivo* by probiotic bacteria. *Gut* **51:** 659–664.
6. Catania, A., V. Gerloni, S. Procaccia, L. Airaghi, M. G. Manfredi, C. Lomater, L. Grossi, and J. M. Lipton. 1994. The anticytokine neuropeptide alpha-melanocyte-stimulating hormone in synovial fluid of patients with rheumatic diseases: Comparisons with other anticytokine molecules. *Neuroimmunomodulation* **1:** 321–328.
7. Catania, A. and J. M. Lipton. 1993. Alpha-Melanocyte stimulating hormone in the modulation of host reactions. *Endocr. Rev.* **14:** 564–576.
8. Colombo, G., R. Buffa, M. T. Bardella, L. Garofalo, A. Carlin, J. M. Lipton, and A. Catania. 2002-2003. Anti-inflammatory effects of alpha-melanocyte stimulating hormone in celiac intestinal mucosa. *Neuroimmunomodulation* **10:** 208–216.
9. Cotta, M. A. and T. R. Whitehead. 1993. Regulation and cloning of the gene encoding amylase activity of the ruminal bacterium *Streptococcus bovis*. *Appl. Environ. Microbiol.* **59:** 189–196.
10. Dieleman, L. A., M. S. Goerres, A. Arends, D. Sprengers, C. Torrice, F. Hoentjen, W. B. Grenther, and R. B. Sartor. 2003. *Lactobacillus GG* prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. *Gut* **52:** 370–376.
11. Dubertret, L., C. Lebreton, and R. Touraine. 1982. Neutrophil studies in psoriatics: *In vivo* migration, phagocytosis and bacterial killing. *J. Invest. Dermatol.* **79:** 74–78.
12. Fichtner-Feigl, S., I. J. Fuss, J. C. Preiss, W. Strober, and A. Kitani. 2005. Treatment of murine Th1- and Th2-mediated inflammatory bowel disease with NF-kappa B decoy oligonucleotides. *J. Clin. Invest.* **115:** 3057–3071.
13. Gantz, I., Y. Konda, T. Tashiro, Y. Shimoto, H. Miwa, G. Munzert, S. J. Watson, J. DelValle, and T. Yamada. 1993. Molecular cloning of a novel melanocortin receptor. *J. Biol. Chem.* **268:** 8246–8250.
14. Gonzalez-Rey, E., N. Varela, A. F. Sheibanie, A. Chorny, D. Ganea, and M. Delgado. 2006. Cortistatin, an antiinflammatory peptide with therapeutic action in inflammatory bowel disease. *Proc. Natl. Acad. Sci. USA* **103:** 4228–4233.
15. Hogaboam, C. M., B. A. Vallance, A. Kumar, C. L. Addison, F. L. Graham, J. Gauldie, and S. M. Collins. 1997. Therapeutic effects of interleukin-4 gene transfer in experimental inflammatory bowel disease. *J. Clin. Invest.* **100:** 2766–2776.
16. Karin, M. and A. Lin. 2002. NF-kappaB at the crossroads of life and death. *Nat. Immunol.* **3:** 221–227.
17. Kojouharoff, G., W. Hans, F. Obermeier, D. N. Männel, T. Andus, J. Schölmerich, V. Gross, and W. Falk. 1997. Neutralization of tumor necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulfate sodium-induced colitis in mice. *Clin. Exp. Immunol.* **107:** 353–358.
18. Krieglstein, C. F., W. H. Cerwinka, A. G. Sprague, F. S. Laroux, M. B. Grisham, V. E. Koteliansky, N. Senninger, D. N. Granger, and A. R. de Fougerolles. 2002. Collagen-binding integrin alpha1beta1 regulates intestinal inflammation in experimental colitis. *J. Clin. Invest.* **110:** 1773–1782.
19. Lee, J. S., H. Poo, D. P. Han, S. P. Hong, K. Kim, M. W. Cho, E. Kim, M. H. Sung, and C. J. Kim. 2006. Mucosal

immunization with surface-displayed severe acute respiratory syndrome coronavirus spike protein on *Lactobacillus casei* induces neutralizing antibodies in mice. *J. Virol.* **80:** 4079–4087.

20. Lipton, J. M., H. Zhao, T. Ichiyama, G. S. Barsh, and A. Catania. 1999. Mechanisms of anti-inflammatory action of alpha-MSH peptides: *In vivo* and *in vitro* evidence. *Ann. N.Y. Acad. Sci.* **885:** 173–182.
21. Luger, T. A., T. Brzoska, T. E. Scholzen, D. H. Kalden, C. Sunderkotter, C. Armstrong, and J. Ansel. 2000. The role of alpha-MSH as a modulator of cutaneous inflammation. *Ann. N.Y. Acad. Sci.* **917:** 232–238.
22. Maaser, C., K. Kannengiesser, C. Specht, A. Lugering, T. Brzoska, T. A. Luger, W. Domschke, and T. Kucharzik. 2006. Crucial role of the melanocortin receptor MC_1R in experimental colitis. *Gut* **55:** 1415–1422.
23. Matsumoto, S., T. Hara, T. Hori, K. Mitsuyama, M. Nagaoka, N. Tomiyasu, A. Suzuki, and M. Sata. 2005. Probiotic *Lactobacillus*-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. *Clin. Exp. Immunol.* **140:** 417–426.
24. Mimura, T., F. Rizzello, U. Helwig, G. Poggioli, S. Schreiber, *et al.* 2004. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* **53:** 108–114.
25. Narita, J., K. Okano, T. Kitao, S. Ishida, T. Sewaki, M. H. Sung, H. Fukuda, and A. Kondo. 2006. Display of alpha-amylase on the surface of *Lactobacillus casei* cells by use of the PgsA anchor protein, and production of lactic acid from starch. *Appl. Environ. Microbiol.* **72:** 269–275.
26. Okayasu. I., S. Hatakeyama, M. Yamada, T. Ohkusa, Y. Inagaki, and R. Nakaya. 1990. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98:** 694–702.
27. Oktar, B. K., F. Ercan, B. C. Yegen, and I. Alican. 2000. The effect of alpha-melanocyte stimulating hormone on colonic inflammation in the rat. *Peptides* **21:** 1271–1277.
28. Papadakis, K. A. and S. R. Targan. 2000. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu. Rev. Med.* **51:** 289–298.
29. Podolsky, D. K. 1991. Inflammatory bowel disease. *N. Engl. J. Med.* **325:** 928–937.
30. Rajora, N., G. Boccoli, D. Burns, S. Sharma, A. P. Catania, and J. M. Lipton. 1997. Alpha-MSH modulates local and circulating tumor necrosis factor-alpha in experimental brain inflammation. *J. Neurosci.* **17:** 2181–2186.
31. Rajora, N., G. Boccoli, A. Catania, and J. M. Lipton. 1997. Alpha-MSH modulates experimental inflammatory bowel disease. *Peptides* **18:** 381–385.
32. Schultz, M., C. Veltkamp, L. A. Dieleman, W. B. Grenther, P. B. Wyrick, S. L. Tonkonogy, and R. B. Sartor. 2002. *Lactobacillus plantarum 299V* in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm. Bowel Dis.* **82:** 71–80.
33. Seegers, J. F. 2002. Lactobacilli as live vaccine delivery vectors: Progress and prospects. *Trends Biotechnol.* **20:** 508–515.
34. Shanahan, F. 2001. Probiotics in inflammatory bowel disease. *Gut* **48:** 609.
35. Siegmund, B., F. Rieder, S. Albrich, K. Wolf, C. Bidlingmaier, G. S. Firestein, *et al.* 2001. Adenosine kinase inhibitor GP515 improves experimental colitis in mice. *J. Pharmacol. Exp. Ther.* **296:** 99–105.
36. Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut. 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289:** 1352–1355.
37. Tien, M. T., S. E. Girardin, B. Regnault, L. Le Bourhis, M. A. Dillies, J. Y. Coppée, R. Bourdet-Sicard, P. J. Sansonetti, and T. Pédron. 2006. Anti-inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells. *J. Immunol.* **176:** 1228–1237.
38. Vowinkel, T., T. J. Kalogeris, M. Mori, C. F. Krieglstein, and D. N. Granger. 2004. Impact of dextran sulfate sodium load on the severity of inflammation in experimental colitis. *Dig. Dis. Sci.* **49:** 556–564.
39. Vowinkel, T., M. Mori, C. F. Krieglstein, J. Russell, F. Saijo, S. Bharwani, *et al.* 2004. Apolipoprotein A-IV inhibits experimental colitis. *J. Clin. Invest.* **114:** 260–269.