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Original Article

Preferential promotion of apoptosis of monocytes by *Lactobacillus casei rhamnosus* soluble factors

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SUMMARY

Background & aims: Inflammatory bowel disease (IBD) is characterized by dense infiltrates of and defective apoptosis by mucosal cell populations. Some probiotics inhibit monocytes' expansion, although mechanisms remain unknown. Supernatants of *Lactobacillus* strains were investigated for inducing apoptosis of monocytes.

Methods: Secreted factors produced by *Latobacillus* strains were tested on human lymphocytes, monocytes and a human monocytic leukemia-cell line (THP-1). Cell death mechanisms were investigated by a variety of methods. Lipopolysaccharide (LPS)-induced proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) and anti-inflammatory TGF-β1 were determined.

Results: Soluble factor(s) from *Lactobacillus casei rhamnosus* strain supernatants (*LcrS*) effectively induced apoptosis of immune cells. These were mainly soluble proteins (MW 5–30 kDa; *Lcr*S_{5-30}). For immune cells, but not human colonic epithelial carcinoma cells (HT-29), pretreatment with *Lcr*S_{5-30} significantly promoted apoptosis via a mitochondrial pathway. *Lcr*S_{5-30} suppressed pro-inflammatory cytokines and induced anti-inflammatory TGF-β1.

Conclusions: Probiotic *Lcr* produced heat-stable molecules (MW range 5–30 kDa) that promoted immune cell apoptosis without affecting intestinal epithelial cells. *Lcr*S_{5-30} triggered apoptosis by a mitochondrial pathway, but not via TGF-β signaling pathway. *Lcr*S_{5-30} also inhibited LPS-induced inflammatory cytokines by immune cells. Thus, *Lcr*S_{5-30} promotes apoptosis of immune cells, and suggests probiotics-based regimens for prevention of IBD.

1. Introduction

Probiotics have been evaluated as an alternative and safe therapeutic approach for inflammatory bowel disease (IBD).[1] Clinical trials suggested that treatments with a multi-species probiotic, VSL#3, including 3 *Bifidobacterium* species, 4 *Lactobacillus* species and *Streptococcus salivarius* ssp. *thermophilus*, were effective for maintaining remission and decreasing relapse rates in IBD patients.[2] Treatment using *Escherichia coli* Nassle 1917 showed remission maintenance for ulcerative colitis (UC).[3] However, conflicting results have been observed for various probiotic strains in clinical use. Treatment with either *Lactobacillus rhamnosus GG* (LGG) or *L. johnsonii* (LA1) did not improve clinical conditions for Crohn's disease (CD) or prevent endoscopic recurrence of CD, respectively.[4,5]

The precise molecular mechanisms by which particular probiotics improve IBD remain unknown. Recent studies suggested that several probiotic strains could have apoptosis inducing capabilities, which would have positive effects on inflammatory responses. *E. coli* Nissle 1917 supernatant induced γδ T cell apoptosis via caspase- and FasL-dependent pathways.[6] In contrast, *Lactobacillus reuteri* secreted factors promoted human myeloid leukemia-derived cells apoptosis by modulation of NF-κB and MAPK signaling in the presence of TNF-α.[7] In addition, soluble proteins produced by LGG can regulate intestinal epithelial cell

Abbreviations: IBD, inflammatory bowel diseases; TGF, transforming growth factor; TNF, tumor necrosis factor.

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proliferation and survival by preventing cytokine-induced apoptosis.[8] These studies illustrated diverse protective effects by different probiotic strains on inflammatory diseases.

IBD is a chronic recurrent inflammatory condition of the gastrointestinal tract. Although its etiology remains unknown, dense infiltrates of activated T cells and macrophages in the terminal ileum and colon mucosa are characteristics of IBD.[9] Defective apoptosis of mucosal T cells, macrophages and monocytes has been recognized as either an initiating event or necessary step in the pathogenesis of IBD.[10] Highly apoptosis-resistant mucosal cells may trigger inflammation, resulting in increased production of inflammatory cytokines (TNF-α, IL-1β, IL-8, etc.), cytolytic enzymes and reactive oxygen species causing tissue injury and clinical consequences.[11,12] Various pharmaceutical treatments, including corticosteroids, antibiotics and immunomodulators, have been examined regarding their capacity to induce apoptosis in gut mucosal effector cells in IBD.[9] However, current therapeutic agents are only moderately effective for long-term treatment and have been associated with potential long-term toxicity.[13] Thus, alternative strategies for treatments of IBD have been devised to resolve adverse effects and to improve the clinical efficacy.

A previous study demonstrated that *Lcr* can control bacterial and gastrointestinal disease,[14] which motivated this investigation on the effectiveness of *Lcr* on host immune mediation. Using human purified monocytes, this study showed the potency of *Lcr* promoted apoptosis of lymphocytes, monocytes and human monocytic leukemia-derived cell lines (THP-1). *Lcr* promoted selected cells apoptosis via a mitochondrial pathway and was strain-specific. In the presence of protein factors (MW 5–30 kDa), *Lcr* suppressed the production of pro-inflammatory cytokines by *E. coli* lipopolysaccharide (LPS)-activated lymphocytes, monocytes and THP-1 cells independent of apoptosis. Moreover, *Lcr*S_{5-30} could induce the production of anti-inflammatory TGF-β1 and TGF-β-independent cell death.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Lactobaciilus* strain, *L. casei rhamnosus* (*Lcr*), was obtained from Antibiophilus® Laboratoires Lyocentre Ltd, Aurillac, France. *L. casei*, and *L. rhamnosus* were provided by Tzu Chi General Hospital (Hualien, Taiwan). All *Lactobacillus* strains were grown at 37 °C in MRS broth (Difco, Sparks, MD, USA) for 48 h until stationary phase and cell-free supernatants were collected by centrifugation at $1000 \times g$ for 10 min at 4 °C. Fresh MRS broth was used as a control in apoptosis experiments and the cytokine assays.

2.2. Bacterial supernatant collection and size exclusion assays

Cell-free supernatant was collected and centrifuged in a filtering device (Amicon Ultra-15; Millipore, Bedford, MA, USA) using MWCO 50 kDa, 30 kDa and 5 kDa ultrafiltration membranes. The supernatant was loaded into 50 kDa ultrafiltration device, $3300 \times g$ for 30 min. The concentrate was the >50 kDa fraction. The <50 kDa fraction was centrifuged at $3300 \times g$ for 30 min using a 30 kDa ultrafiltration membrane. The upper layer was the 30–50 kDa fraction. The 5–30 kDa fraction was obtained by applying the <30 kDa fraction to 5 kDa ultrafiltration device. The filtrate was the <5 kDa fraction. Each fraction was filtered through a 0.22-μm membrane (Millipore) and kept at 4 °C before use. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

2.3. Denaturation of bacterial supernatant proteins

The supernatant was mixed with 1 mg/ml trypsin or proteinase K (Sigma–Aldrich Co. Ltd., Poole, UK) at room temperature overnight. The supernatant was heated to 100 °C for 30 min prior to an intended assay.

2.4. Cell culture

THP-1 cells (human monocytic leukemia-derived cell line) and HT-29 cells (human colonic epithelial carcinoma cell line) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). THP-1 cells were maintained in Falcon 100×20 mm plastic culture flasks (Becton Dickinson Labware, Oxnard, CA, USA) in RPMI-1640 medium (Gibco, Invitrogen, Paisley, UK), supplemented with 10% fetal bovine serum (Gibco, Invitrogen), L-glutamine (200 mmol/l), 2-mercapto-ethanol (5×10^{-5} mol/l) (Sigma–Aldrich), 0.1 mg/ml of streptomycin sulfate (Sigma–Aldrich), 0.1 mg/ml of penicillin (Brittania Pharmaceuticals Ltd., Poole, UK). HT-29 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for varying times.

2.5. Isolation PBMCs and purification of lymphocytes and monocytes

Peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors and were prepared by density gradient centrifugation of blood over Histopaque-1077 (Sigma–Aldrich). PBMCs were separated into CD14+ monocytes and lymphocyte subsets by immunomagnetic selection with CD14 MicroBeads kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) used according to the manufacturer's instructions. Flow cytometry analysis indicated the purity of monocyte preparation from PBMCs following immunomagnetic selection with anti-CD14 was >95%. A highly enriched population of lymphocytes was obtained from the CD14-depleted cell fraction. Cell preparations were aliquoted to 1×10^6 cells/ml in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum (Gibco Invitrogen), 0.1 mg/ml of streptomycin sulfate (Sigma–Aldrich), 0.1 mg/ml of penicillin (Brittania Pharmaceuticals Ltd) and 100 mM L-glutamine (Sigma–Aldrich). Cell culture was at 37 °C in a humidified atmosphere with 5% CO_2 for varying times.

2.6. Annexin V staining

Lymphocytes, monocytes and THP-1 cells (10^6 per test) were collected after incubation with *Lcr*S, 3.125–25 μg/ml for varying times. After washing twice with PBS, the cells were resuspended in Annexin V binding buffer (10 mM HEPES–NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$). After centrifugation, the cells were incubated in 500 μl of the same buffer containing 1.25 μl FITC-conjugated Annexin V (BD PharMingen, San Diego, CA, USA) at room temperature for 15 min in the dark. Data acquisition and analysis used a Becton–Dickinson FACSan flow cytometer (Franklin Lakes, NJ, USA) with CellQuest software (Becton–Dickinson, Oxford, UK).

2.7. TUNEL assay

THP-1 cells were cultured in plates and treated with 10 μM taxol or 3.125–25 μg/ml of the *Lcr*S_{5-30} for 24 h. After PBS wash, 1×10^6 cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature and permeabilized with 80% ethanol at −20 °C. DNA fragments of apoptotic cells were labeled with 57 μL biotin and 3 μL

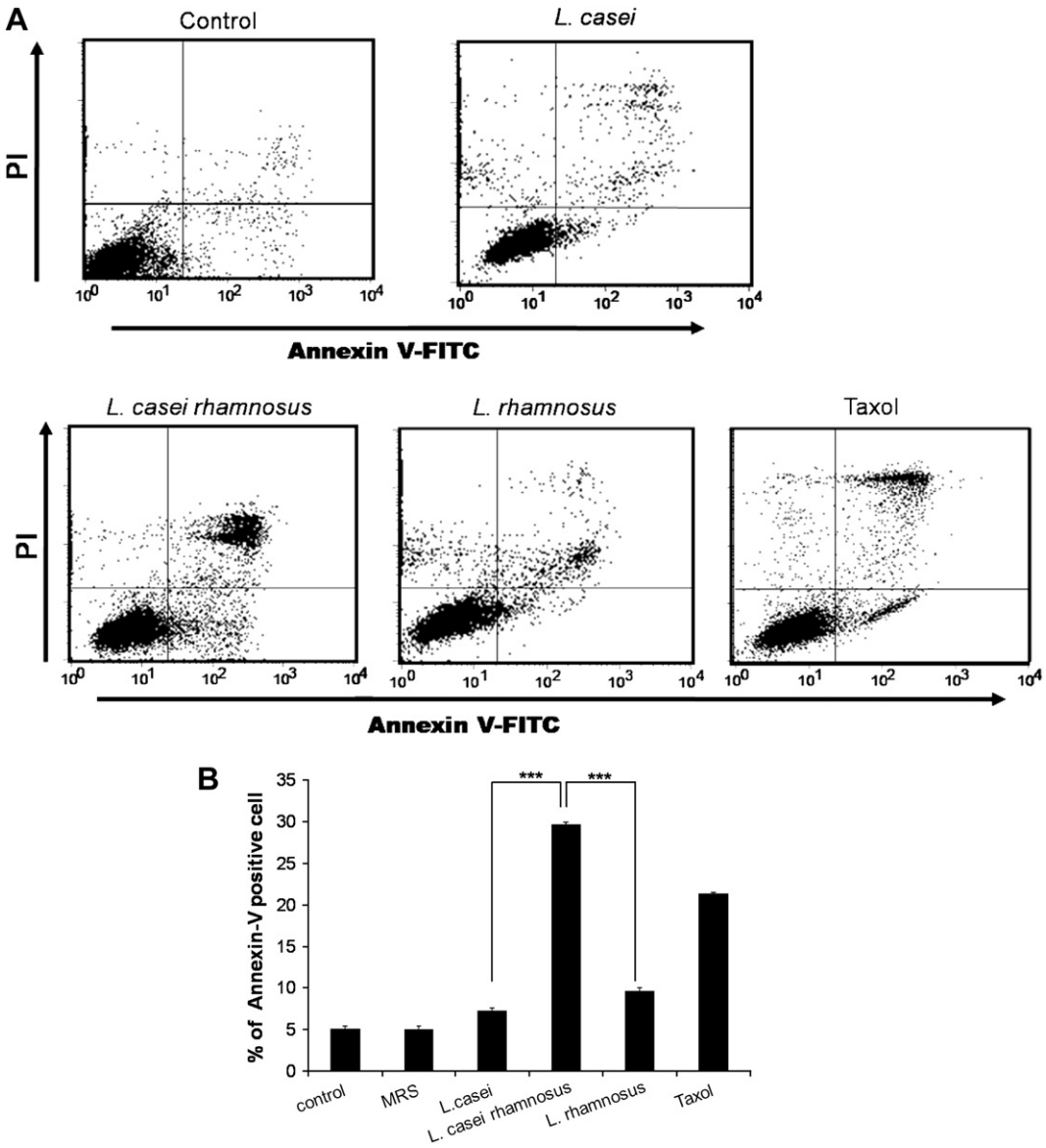

Fig. 1. Strain-dependent promotion of apoptosis of THP-1 cells in the absence or presence of probiotic-derived secreted factors. *Lcr*S significantly increased the numbers of Annexin V positive cells, but fewer were found for *L. casei* and *L. rhamnosus*. Apoptotic THP-1 cells following exposure to 25 μg/ml probiotic supernatants were determined by double staining with Annexin V-FITC and Propidium iodide (PI). Cells that were Annexin V positive and PI negative were early apoptosis cells. Cells positive both for Annexin V and PI represented cells in late apoptosis. Results representative of typical donors (panel A) and mean ± standard error of the mean (panel B) for 3 different experiments. Control = complete RPMI cell culture medium. MRS = bacterial culture medium. 1 μM Taxol was a positive control. $^{***}P < 0.001$ for change versus *Lcr*S.

catalyzed by terminal deoxynucleotidyl transferase (TdT) per sample for 2 h. Biotinylated nucleotides were detected using 100 μL streptavidin–horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacted with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Slides were counterstained with methyl green for morphological evaluation and characterization of normal and apoptotic cells using light microscopy. A total of 200 cells per sample were analyzed, and the numbers of apoptotic cells were expressed as percentages of the total cells.

2.8. Assessment of mitochondrial membrane potential ($\Delta\Psi m$)

$\Delta\Psi m$ was measured using the lipophilic cation JC-1, which has potential-dependent accumulation in mitochondria indicated by a fluorescence emission shift from green (JC-1 in monomeric form, 527 nm) to red (JC-1 in aggregative form, 590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. Purified lymphocytes, monocytes and THP-1 cells were aliquoted to 5×10^5 cells/ml in supplemented RPMI medium and incubated with or without $LcrS_{5-30}$ for varying time intervals and doses ranging from 3.125 to 25 μg/ml at 37 °C in the presence of 5% CO_2. Cells were then washed twice with PBS prior to staining with 1 μM JC-1 in dimethyl sulfoxide (DMSO) (Gibco Invitrogen) for 30 min in the dark at 37 °C, and immediately analyzed with a Becton–Dickinson FACSan flow cytometer at 488 nm excitation.

2.9. Detection of activated caspase-9

Lymphocytes, monocytes and THP-1 cells (5×10^5 cells/ml) were collected after incubation with $LcrS_{5-30}$ for varying times and

at doses ranging from 3.125 to 25 μg/ml at 37 °C in the presence of 5% CO_2. After incubation, cells were fixed with 4% formaldehyde in PBS for 5 min at room temperature, followed by permeabilization with saponin buffer (0.04% saponin, 50 mM glucose, 0.1% sodium azide) in PBS. Intracellular active caspase-9 subunits were detected by incubation with FITC-conjugated anti-active human caspase-9 antibody (BD Pharmingen) in the presence of rabbit serum (Sigma–Aldrich) to block nonspecific binding. Substrate cleavage of released free FITC fluorescence intensities were recorded with a Becton-Dickinson FACS-Calibur flow cytometer with excitation wavelength at 488 nm and emission wavelength at 520 nm.

2.10. Western blot analysis

Monocytes and THP-1 cells were harvested from cultures after treatment with the *Lcr*S_{5-30} for varying time intervals, 3–24 h. Cells were lysed with buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% NaN_3, and a protease inhibitor mixture (Sigma–Aldrich). After 1 freeze-thaw cycle, cell lysates were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatants were collected and boiled in the sample buffer for 5 min. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore), blocked overnight at 4 °C in PBS-T (PBS plus 0.5% Tween 20) containing 10% skim milk, and probed with Abs against cytochrome *c*, pro-caspase 3, active caspase 3, and β-actin (Calbiochem, San Diego, CA, USA) for 1 h at room temperature. After washes with PBS-T, blots were incubated with a 1/5000 dilution of HRP conjugated goat anti-mouse or anti-rabbit IgG (Calbiochem) for 1 h at room temperature. Protein bands were developed using ECL Plus Western Blotting Detection System (Pierce).

2.11. RT-PCR

Total cellular RNA was extracted using an Ultraspec-II RNA isolation system (Biotec, Houston, TX, USA) following the manufacturer's instructions. The concentration of RNA was quantitated by spectrophotometry at 260 nm. cDNA in a total volume of 20 μL was prepared after reverse transcription of cellular RNA (5 μg) with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) using a 20-mer of oligo(dT) as the primer. cDNA (3 μL) was added to PCR buffer containing primers at 1.5 μM each, $MgCl_2$ (1.5 mM), dNTPs (0.2 mM each), and 1 U of *Taq* DNA polymerase (Promega) in a total reaction volume of 50 μL. The oligonucleotide primers for human Bcl-2 (5′-ACAACATCGCCCTGTGGATGA-3′ and 5′-ATAGCTGATTCGACGTTTTGCC-3′), Bax (5′-GGAATTCTGACGGCAACTTCAACTGGG-3′ and 5′-GGAATTCTTCCAGATGGTGAGCGAGG-3′), and β-actin (5′-AGCGGGAA ATCGTCGTG-3′ and 5′-CAGGGTACATGGTGGTGGTGCC-3′) were used according to previously published sequences.[15] Thirty-five cycles were used for Bcl-2 and Bax, and 30 cycles for β-actin (95 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min) using a PCR controller (GeneAmp PCR System 2400; PerkinElmer, Wellesley, MA, USA). The PCR products were separated by 1% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, and viewed with UV light.

2.12. Cytokine assays

Lymphocytes, monocytes and THP-1 cells, 5×10^5 cells/ml, were stimulated with 1 μM LPS and 25 μg/ml *Lcr*S_{5-30}. After 24 h, culture supernatants were collected, centrifuged and stored at −20 °C for cytokines and chemokine analysis. Quantitative human IL-1β, IL-6, IL-8, TNF-α and TGF-β1-specific ELISAs (DuoSet, R & D systems, Minneapolis, MN, USA) were performed in a 96-well plate and detected at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

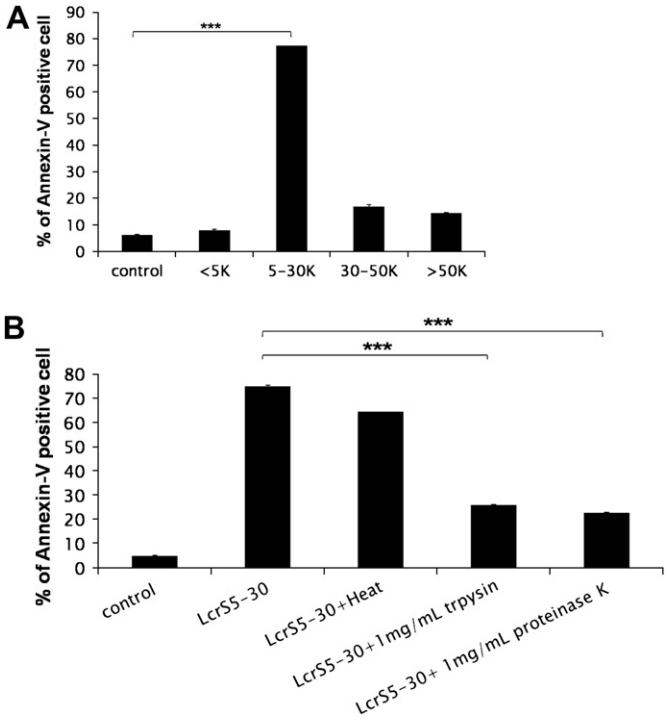

Fig. 2. Partial characterization of factors promoting THP-1 apoptosis. THP-1 cells were stimulated with 25 μg/ml of 4 different fractions (panel A). THP-1 cells were stimulated with *Lcr*S_{5-30} that had either been boiled for 30 min or treated with 1 mg/ml trypsin or proteinase K. After 24 h stimulation, apoptotic THP-1 cells following exposure to 25 μg/ml *Lcr*S_{5-30} were determined by double staining with Annexin-V FITC and PI (panel B). Each bar represents mean ± standard error of the mean of 3 individual experiments. ***$P < 0.001$ for change versus control.

2.13. Statistical analysis

All experiments were performed at least 3 times, each time in triplicate. Data were analyzed by multivariate ANOVA test. If a significant difference was found, a least significant differences (LSD) multiple comparison test was used to identify significant groups. Statistical analyses used The Statistical Software Package for the Social Sciences, version 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Probiotic Lcr preferentially induces apoptosis of a human monocytic leukemia-derived cell line

Following incubation of THP-1 cells with *Lactobacillus* conditioned media, the relative apoptosis promoting activities of probiotics were evaluated by Annexin-V FITC and PI staining in cell-free culture supernatants followed by flow cytometry. The capabilities of *Lactobacillus* strains' supernatants to induce THP-1 cell apoptosis were determining by culturing the cells with 25 μg/ml of each supernatant for 24 h. *Lcr*S significantly increased the proportion of Annexin-V positive cells (24.58%; $P < 0.001$), whereas the *L. casei* and *L. rhamnosus* strains induced limited Annexin-V positive staining (2.17% and 4.53%, respectively; Fig. 1).

3.2. Partial characterization of the Lcr apoptosis-inducing factor(s)

*Lcr*S was divided into 4 four fractions based on molecular weight using ultrafiltration membranes: <5 kDa, 5–30 kDa,

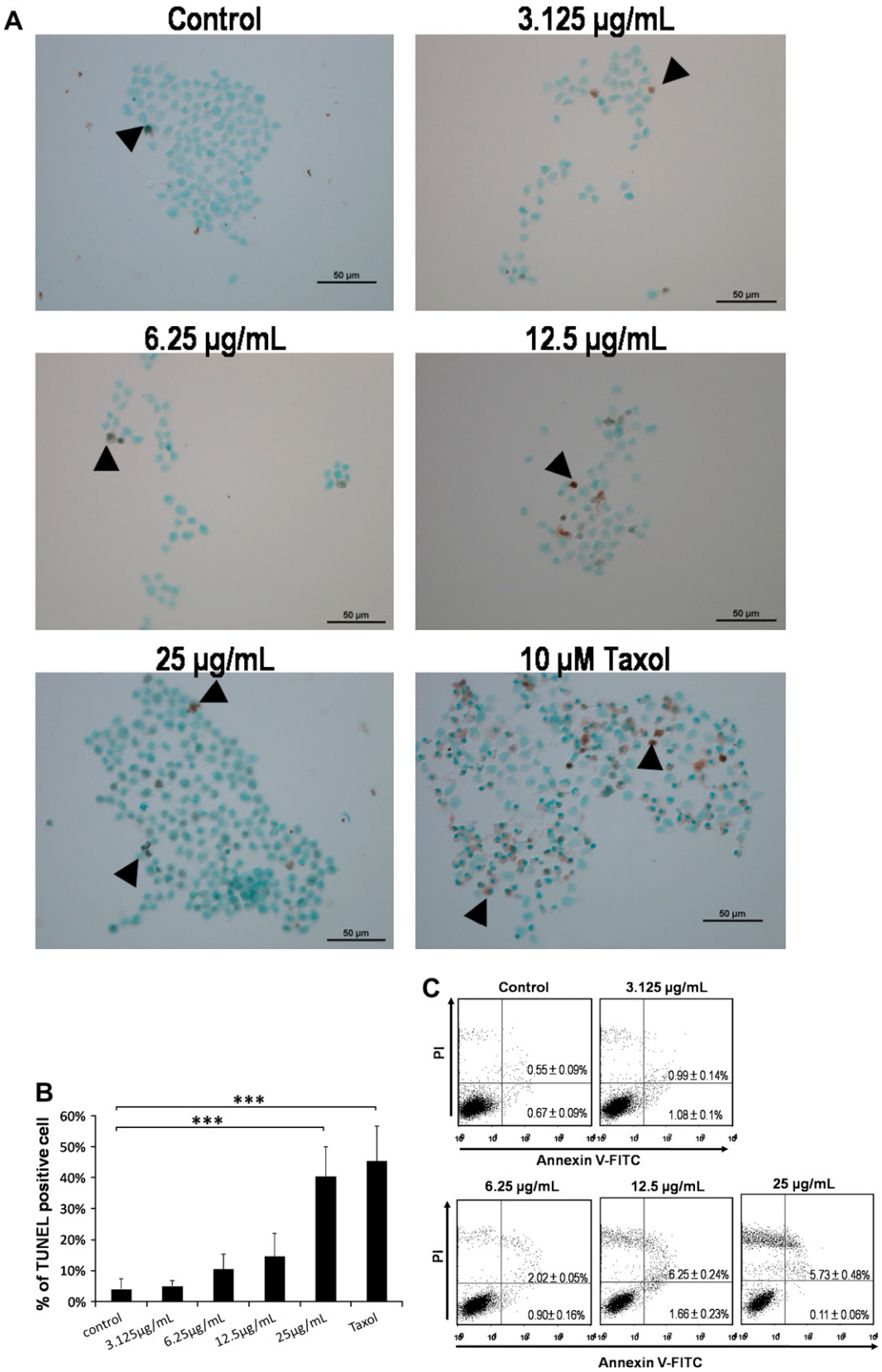

Fig. 3. *Lcr*S_{5-30} promoted apoptosis of monocytes, but not intestinal epithelial cells. THP-1 cells (panels A and B) and HT-29 cells (panel C) were treated with varying concentrations of *Lcr*S_{5-30} or 10 μM Taxol as a positive control for 24 h. TUNEL staining was observed by light microscope. Arrows indicate representative apoptotic cells (panel A). The percentage of cells that underwent apoptosis from a representative experiment (panel B). Apoptotic HT-29 cells exposed to *Lcr*S_{5-30} were determined by Annexin V-FITC and PI double staining followed by flow cytometric analysis (panel C). All experiments were performed on at least 3 separate occasions. $^{***}P < 0.001$ for change versus control.

30–50 kDa and >50 kDa. These fractions induced, respectively, 8.03%, 77.36%, 16.91% and 14.43%Annexin-V positive cells. The *Lcr*S$_{5-30}$ fraction promoted apoptosis more effectively than the others ($P < 0.001$, Fig. 2A).

Then, *Lcr*S$_{5-30}$ was subjected to various treatments in order to identify the nature of the compounds responsible for the promotion of apoptosis of THP-1 cells. Heating did not decrease the apoptosis-promoting effect, suggesting that the apoptosis-promoting compounds were heat-stable. Proteinase K and trypsin treatments reduced Annexin-V positive cells by 69.8% and 65.53%, respectively, compared to an untreated fraction ($P < 0.001$). These results suggested that proteinaceous compounds or domain(s) may be involved with promoting apoptosis (Fig. 2B).

3.3. Selective apoptosis promotion by *Lcr*S$_{5-30}$ on monocytes, but not intestinal epithelial cells

THP-1 cells were analyzed by TUNEL assay, while the human colonic epithelial carcinoma cell line (HT-29) cells were analyzed by Annexin V-FITC and PI double staining using flow cytometry. Exposure of THP-1 cells to varying concentrations of *Lcr*S$_{5-30}$ (3.125–25 μg/ml) led to dose-dependent apoptosis induction (Fig. 3A and 3B). In contrast, the majority of HT-29 cells did not undergo apoptosis after exposures to the same concentrations of *Lcr*S$_{5-30}$ (Fig. 3C).

3.4. Dose- and time dependence of *Lcr*S$_{5-30}$-induced cytotoxicity

In order to quantify apoptosis induction of immune cells by *Lcr*S$_{5-30}$, both monocytes and lymphocytes were purified from PBMC samples and subjected to similar treatments. Double staining with Annexin-V FITC and PI confirmed time- and dose-dependent apoptosis promotion of lymphocytes, monocytes and THP-1 cells by *Lcr*S$_{5-30}$. To determine the timing of apoptosis induced by *Lcr*S$_{5-30}$, cultured cells were treated with 25 μg/ml of *Lcr*S$_{5-30}$ for varying times. Flow cytometry analyses showed that *Lcr*S$_{5-30}$ induced monocytes and THP-1 apoptosis in a time-dependent manner. *Lcr*S$_{5-30}$ also promoted apoptosis of lymphocytes at 12–24 h after treatment (Fig. 4A).

To determine the dose of *Lcr*S$_{5-30}$ required to induce apoptosis, cells were treated with different concentrations of *Lcr*S$_{5-30}$ for 24 h. These results showed that 3.125 μg/ml of *Lcr*S$_{5-30}$ effectively induced apoptosis of monocytes and lymphocytes. By comparison, 25 μg/ml of *Lcr*S$_{5-30}$ promoted significant apoptosis of THP-1 cells (Fig. 4B).

3.5. Apoptosis of monocytes by *Lcr*S$_{5-30}$ via a mitochondrial pathway

The loss of mitochondrial membrane potential ($\Delta\Psi m$) is an important event in apoptosis. The carbocyanine dye JC-1 detects changes in $\Delta\Psi m$ due to its dual emission characteristics.[16] A two-parameter fluorescence display of JC-1-stained monocytes showed that most of the cells emitted relatively lower levels of green fluorescence, while a subpopulation exhibited reduced JC-1 aggregation and an increase in green fluorescence emission, indicating a decrease of $\Delta\Psi m$. Exposure of purified monocytes to 3.125 μg/ml and 6.25 μg/ml of *Lcr*S$_{5-30}$ for 24 h did not induce significant changes in emission of JC-1 (11.59% and 13.71%, respectively; % of depolarized $\Delta\Psi m$ cells). However, 12.5 μg/ml *Lcr*S$_{5-30}$ strongly induced depolarized $\Delta\Psi m$ cells (56.43%, $P < 0.001$, Fig. 5A).

In addition, a high correlation has been previously shown between $\Delta\Psi m$ in isolated mitochondria and fluorescence ratio (mean red fluorescence intensity/mean green fluorescence intensity corresponding to the FL2/FL1 ratio). Thus, to determine the

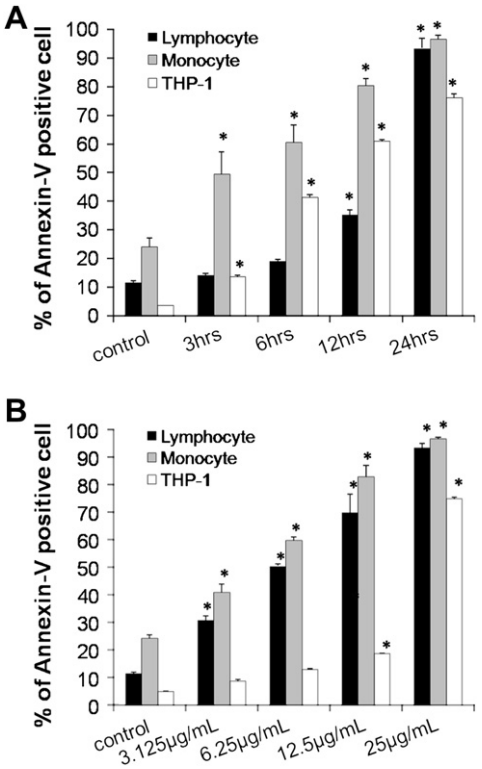

Fig. 4. Incubation with *Lcr*S$_{5-30}$ increased the numbers of Annexin V positive cells in a time- and dose-dependent manner. Lymphocytes (■), monocytes (▒) or THP-1 cells (□) (1×10^6 per test) were collected after incubation with *Lcr*S$_{5-30}$ for varying times (panel A) and for doses ranging from 3.125 to 25 μg/ml (panel B). Cell apoptosis was determined by double staining with Annexin V-FITC and PI. Results are mean ± standard error of the mean from triplicate cultures. *$P < 0.01$ for change versus control.

$\Delta\Psi m$ values for lymphocytes, monocytes and THP-1 samples after *Lcr*S$_{5-30}$ treatment, the fluorescence ratios were evaluated (Fig. 5B). The FL2/FL1 ratio was decreased as early as 3 h after *Lcr*S$_{5-30}$ treatment. For the tested cells, the maximum loss of $\Delta\Psi m$ was reached at approximately 3 h, and remained at this level until 12 h after treatment.

To understand the modulation in the apoptotic signaling pathway by *Lcr*S$_{5-30}$, expressions of the Bcl-2 family proteins Bcl-2 and Bax were analyzed by RT-PCR (Fig. 6A). After treatment with *Lcr*S$_{5-30}$ for monocytes and THP-1 cells, the mRNA levels of Bcl-2 decreased, whereas that of Bax mRNA expression increased in a time-dependent manner (Fig. 6A). The release of cytochrome *c* from mitochondria to cytoplasm was determined by Western blot (Fig. 6B). Also, the cleavages of pro-caspase 9 and pro-caspase 3 into their active forms were detected 3–24 h after *Lcr*S$_{5-30}$ treatment (Fig. 6B and C). These results indicated that *Lcr*S$_{5-30}$ regulated the apoptosis of monocytes and THP-1 cells via expressions of mRNAs, including Bcl-2 and Bax, and proteins, including cytochrome *c*, caspase 9 and caspase 3, by a mitochondrial pathway.

3.6. Inhibition of LPS-induced inflammatory cytokines after treatment with *Lcr*S$_{5-30}$

The apoptosis-inducing effects on pro-inflammatory cells by *Lcr*S$_{5-30}$ implied its potential for regulating the secretions of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α). Lymphocytes, monocytes and THP-1 cells were treated with *E. coli*-derived LPS and *Lcr*S$_{5-30}$, and the changes of inflammatory cytokines secretions were evaluated by ELISA (Fig. 7). As expected, LPS-treated cells

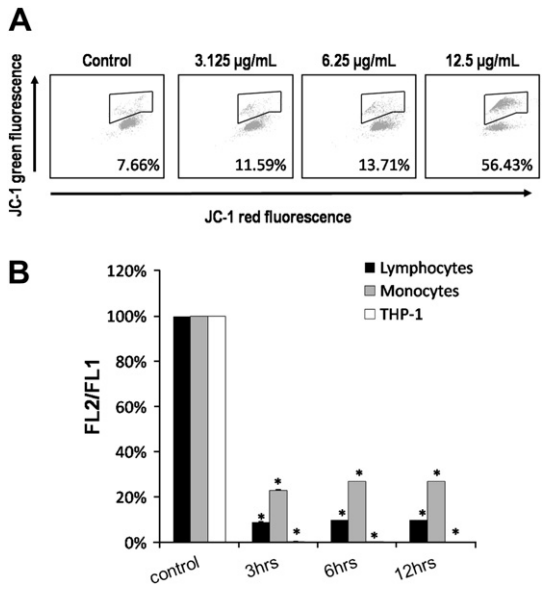

Fig. 5. Cytofluorometric analysis of mitochondrial membrane potential ($\Delta\Psi m$). One representative analysis of $\Delta\Psi m$ in monocytes after stimulation with varying concentrations of *Lcr*S_{5-30} (panel A). Respective percentages of cells with depolarized mitochondria (%$\Delta\Psi m$) are indicated in the upper box of each group (7.66, 11.59, 13.71 and 56.43%, respectively). Thus, a decrease in $\Delta\Psi m$ corresponds to an increase in percent of monocytes %$\Delta\Psi m$. JC-1 red fluorescence/JC-1 green fluorescence ratio for lymphocytes, monocytes and THP-1 with depolarized $\Delta\Psi m$, with or without the *Lcr*S_{5-30}, for varying times (panel B). $^*P < 0.01$ for change versus control.

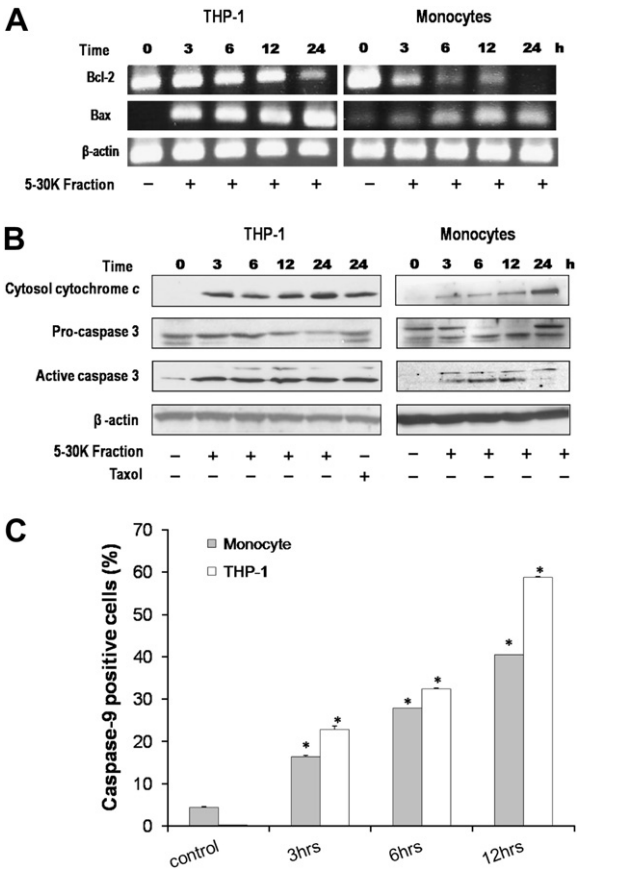

Fig. 6. *Lcr*S_{5-30} causes decreased Bcl-2 expression, increased Bax and caspase 9 expressions, cytochrome *c* (Cyt c) release and caspase 3 activation. Monocytes and THP-1 cells (1×10^6) were treated with or without 20 μg/ml of *Lcr*S_{5-30} for indicated times. mRNA was detected by RT-PCR (panel A), and the protein levels were determined by Western blot (panel B). Both the mRNA and protein expressions of actin were internal controls. Cells were incubated with FITC-conjugated anti-active caspase-9 antibody and analysis by flow cytometry (panel C). Monocytes and THP-1 cells exhibiting positive intracellular active caspase-9 fluorescence were enumerated, and the results are expressed as a percentage of the total number of cells analyzed. Results are mean ± standard error of the mean of 3 individual experiments. $^*P < 0.01$ for change versus control.

showed elevated levels of inflammatory cytokines. However, cytokines expressions were markedly reduced after simultaneous incubation with *Lcr*S_{5-30} ($P < 0.01$, Fig. 7). These experiments suggested that *Lcr*S_{5-30} was capable of downregulating the secretion of pro-inflammatory cytokines.

3.7. *Lcr*S_{5-30} induces TGF-β production and promotes TGF-β-independent apoptosis

TGF-β1 is a key regulatory cytokine involved in anti-inflammation that counteracts IL-1 and TNF-α and modulates cellular functions, such as homing, cellular adhesion, chemotaxis and T-cell homeostatic regulation.[17] In contrast to the pro-inflammatory cytokines described above, dramatic increases of TGF-β1 levels were observed following treatment with *Lcr*S_{5-30} (Fig. 8A).

TGF-β regulates a wide array of biological functions, including apoptosis.[18] TGF-β production was increased by *Lcr*S_{5-30} treated THP-1 cells. It was reasonable to postulate that apoptosis might be triggered by TGF-β rather than *Lcr*S_{5-30}. To confirm this assumption, a pharmacological inhibitor, SB431542, was applied. The percentages of apoptotic cells in 10 ng/ml and 1 ng/ml TGF-β exposed preparations were 18.9 ± 0.44% and 16.57 ± 0.67%, respectively (Fig. 8B). Treatments with 12.5 μg/ml and 25 μg/ml of *Lcr*S_{5-30} induced dissimilar levels of apoptosis of THP-1 cells, which were 26.35 ± 0.77% and 74.96 ± 0.38%, respectively. One ng/ml TGF-β and 12.5 μg/ml *Lcr*S_{5-30} induced apoptosis were SB431542 sensitive. Nevertheless, 25 μg/ml of *Lcr*S_{5-30} induced dramatic changes in the levels of apoptosis of THP-1 cells, and SB431542 could not reverse this phenomenon (Fig. 8B). Collectively, these results indicated that *Lcr*S_{5-30} was capable of inducing TGF-β-independent cell death.

4. Discussion

In the present study, several *Lactobacillus* strains were investigated for their differential capabilities to promote apoptosis of THP-1 cells. *Lcr* exhibited more potent apoptosis inducing capability than the *L. casei* and the *L. rhamnosus* strains. Probiotics, as preventative or therapeutic agents against IBD, are an attractive, alternative approach for the attenuation of mucosal inflammation. Many clinical studies during recent decades demonstrated that probiotic species possessed beneficial effects for IBD.[1–3] However, inconsistency among some results might have resulted from differences in probiotic species or strains. These inconsistencies have drawn attention to understanding the mechanisms of probiosis by specific strains for possible clinical applications.

Initial characterization of the apoptosis inducing factor(s) in *Lcr*S employed MWCO ultrafiltration. The apoptosis inducing factor(s) were present in the 5–30 kDa fraction. Several studies have described apoptosis promotion via factor(s) produced by probiotics, such as for human breast cancer cells by fermented soy milk,[19] human γδ T cells by *E. coli* Nissle 1917 supernatant[6] and for human myeloid leukemia-derived cells by *L. reuteri*.[7] The identities of these factor(s) have not been determined. The *Lcr*-derived apoptosis-inducing factor(s) are small heat stable proteins or peptides components, based on results of heat inactivation and protease treatment experiments.

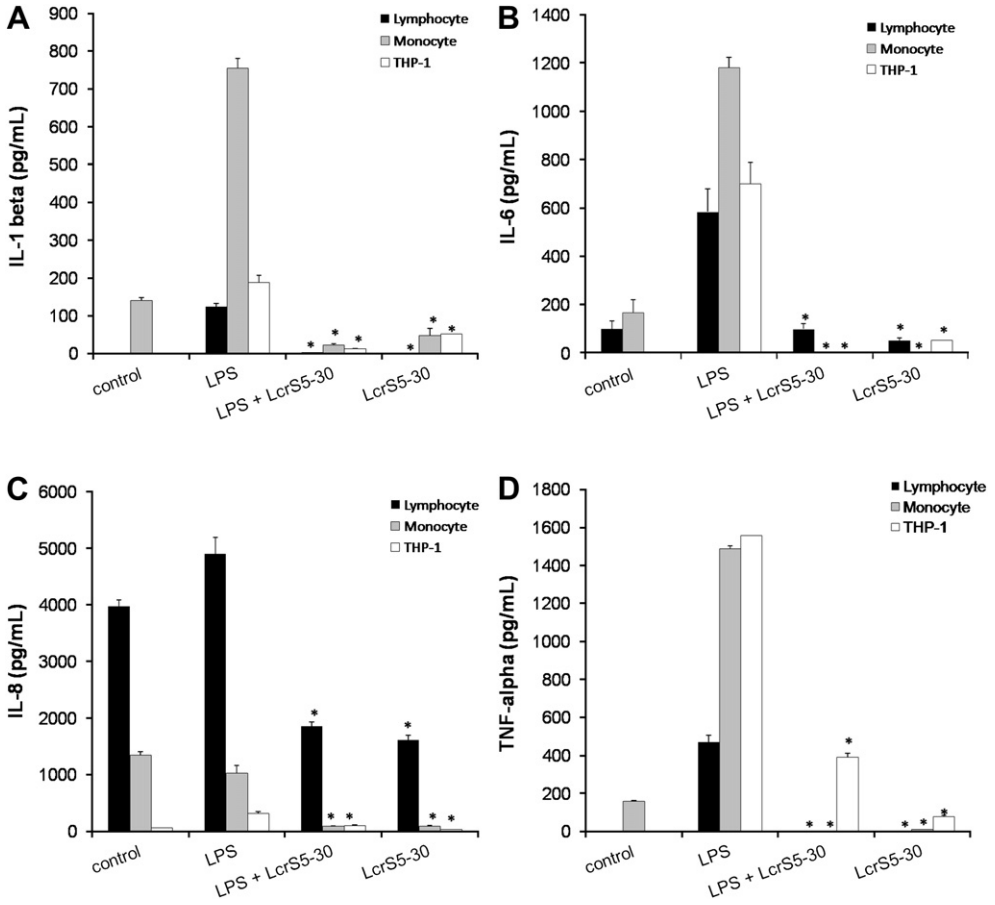

Fig. 7. Inhibition of cytokine production by *Lcr*S_{5-30} by lymphocytes, monocytes and THP-1 cells. Cell culture bioassay were performed by stimulating lymphocytes (■), monocytes (▩) or THP-1 cells (□) with *E. coli*-derived LPS and 25 μg/ml of *Lcr*S_{5-30}. Human IL-1β (panel A), IL-6 (panel B), IL-8 (panel C) and TNF-α (panel D) cytokine were determined in culture supernatants by sandwich ELISA following cell culture. Each bar represents mean ± standard error of the mean of 3 individual experiments. *$P < 0.01$ for change versus LPS.

Pathologically, IBD is characterized by a high density of mucosal cells within the inflamed tissues that mainly consist of activated T cells, peripheral blood neutrophils and monocytes/macrophages.[9] Extensive studies in recent years have shown that activation and increased survival time of leucocytes might contribute to the severity of intestinal inflammation and clinical relapses in both CD and UC.[10] Activation-induced cell death (AICD) is an important mechanism to limit the number of active monocytes and lymphocytes, and to terminate an immune response. *Lcr*S_{5-30} was effective for promoting lymphocyte/monocyte/THP-1 cell apoptosis in both dose- and time-dependent manners.

Despite a paucity of data regarding probiotics for promoting apoptosis of immune effector cells as an alternative IBD therapy, experiments with γδ T cell going through programmed cell death by *E. coli* Nissle and human myeloid leukemia-derived cells proceeding through TNF-induced apoptosis by *L. reuteri* provided a rational basis for an apoptosis-inducing strategy for IBD therapy. Our study with monocytes showed that 12.5 μg/ml and 25 μg/ml of *Lcr*S_{5-30} could disrupt the mitochondrial membrane potential, and apoptotic cell death was induced within 3 h following exposure to 25 μg/ml of *Lcr*S_{5-30}. Moreover, the increase of the Bax/Bcl-2 ratio, release of cytochrome *c*, and activation of caspase-9 and caspase-3, showed that *Lcr*S_{5-30} is capable of inducing monocytes apoptosis via a mitochondrial pathway.

An anti-TNF antibody, infliximab, is an FDA-approved treatment for CD and UC. Several studies demonstrated that infliximab induced monocytes apoptosis[20] and a loss of CD68+ monocytes, as well as CD4+ and CD8+ T lymphocytes, in the lamina propria.[21] Also, infliximab exerted killing activity on human peripheral blood T cells by as much as 50.6% after 18 h culture with 5 μg/mlinfliximab.[22] In our study, *Lcr*S_{5-30} promoted apoptosis of lymphocytes, monocytes and THP-1 cells by as much as 93%, 97% and 74%, respectively. The induction of apoptosis did not require the Fas/FasL signal transduction pathway. Rather, it involved upregulation of Bax/Bak followed by mitochondrial release of cytochrome *c*.[22] Interestingly, the extents of immune effector cells apoptosis induced by infliximab and probiotic *Lcr* were similar. Also, given the safety history of probiotics, *Lcr* could be a useful adjunctive treatment for IBD patients.

The loss of intestinal epithelial cell (IEC) function, and subsequent changes in epithelial tight junction protein expressions and IEC apoptosis are also critical components for the initiation and perpetuation of IBD.[23] Probiotics have been investigated for protective effects by regulating IEC survival for treating and preventing intestinal inflammation. *In vitro* results indicated that LGG reduced intestinal epithelial apoptosis by upregulating the expressions of anti-apoptotic and cytoprotective genes.[24] Also, soluble factors from LGG stimulated anti-apoptotic Akt activation and prevented cytokine-mediated apoptosis.[8] In this study, *Lcr*S_{5-30} was quite unique in that it promoted apoptosis of monocyte-like cells, but not of IEC. These results are consistent with previous studies in which probiotics could protect intestinal integrity by promoting survival of IEC.

LcrS_{5-30} possessed potent inhibitor(s) for LPS induced pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and chemokine, IL-8. The suppression of IL-1β, IL-6, TNF-α and IL-8 suggested that

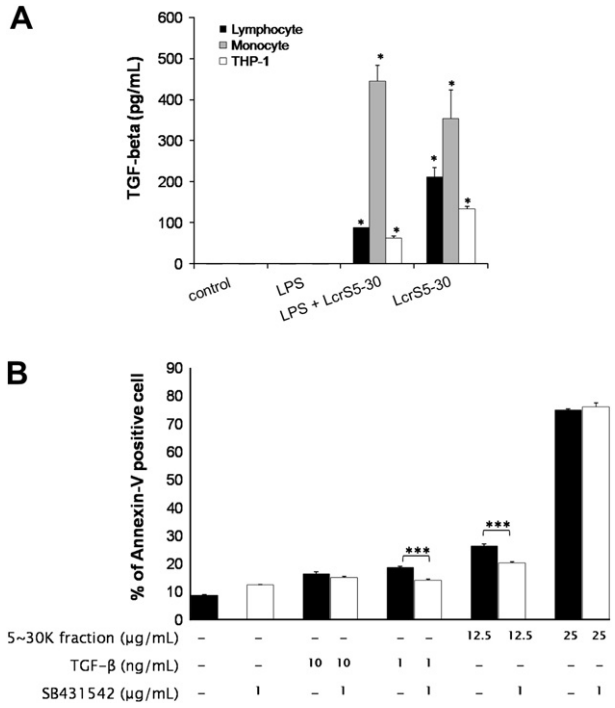

Fig. 8. *Lcr*S_{5-30} induced TGF-β1 production and promoted TGF-β independent apoptosis. Human TGF-β1 quantities were determined by TGF-β1 specific ELISA in culture supernatants following lymphocytes (■), monocytes (▩) or THP-1 cells (□) culture (panel A). THP-1 cells were treated with TGF-β1 or *Lcr*S_{5-30} in the absence or presence of 1 μg/ml of TGF-β inhibitor, SB431542, for 24 h (panel B). Cell apoptosis was determined by double staining with Annexin V-FITC and PI. T Results are mean ± standard error of the mean from triplicate cultures. $^{*}P < 0.01$ for change versus LPS. $^{***}P < 0.001$ for change versus 1 ng/ml SB431542 versus the respective control.

LcrS_{5-30} might concomitantly suppress lymphocyte/monocyte/THP-1 chemotaxis and cellular activation. TNF-α is a crucial proinflammatory cytokine in various inflammatory disorders. Blocking of TNF-α is efficient for the treatment of patients with CD and UC.[25] Interestingly, studies have indicated that apoptosis was indeed linked to the clinical efficacies of various anti-TNF agents. TNF-α inhibitors, such as infliximab and adalimumab, have shown clinical efficacy for IBD and exerted strong apoptotic effects.[21] However, another anti-TNF agent, etanercept, did not establish a positive influence on the course of CD[26] due to a failed apoptosis inducing ability in monocytes and lymphocytes. Our results indicated that *Lcr*S_{5-30} possessed both TNF-α suppression and apoptosis promotion capabilities, as seen with infliximab.

TGF-β is a potent anti-inflammatory cytokine and has a vital role for suppressing the activation and proliferation of inflammatory cells.[27] Also, TGF-β has been implicated for an essential role in disease remission by promoting the maturation of intestinal epithelial cells and for healing wounds and ulcers.[28] *L. paracasei* has been reported to induce populations of regulatory CD4+ T cells, which produce high levels of modulatory cytokines, IL-10 and TGF-β.[29] *Lactobacilli* modulate cytokine production in bone-marrow-derived dendritic cells with a net effect of altering overall cytokine profiles in a species-dependent manner.[30] One study also showed that pediatric IBD patients in remission compared to those with active disease had higher levels of TGF-β1.[28] In this study, because *Lcr*S_{5-30} could induce high TGF-β1 production, but not IL-10 (data not shown), by lymphocytes, monocytes, and THP-1 cells implied that *Lcr*S_{5-30} altered the balance between pro-inflammatory and anti-inflammatory cytokines, and highlights their important immunomodulatory roles in inflammatory diseases.

TGF-β can trigger apoptosis in myeloid leukemia cells.[18] SB-431542, a specific inhibitor of TβRI, inhibits the TGF-β-induced apoptosis in several cell types by blocking TGF-β signaling. Upregulated TGF-β production promotes monocytes apoptosis and contributes to the prevention of tissue injury. It was important to confirm the consequences of TGF-β production in regulating the apoptosis of monocytes following *Lcr*S_{5-30} treatment. SB-431542 significantly blocked TGF-β1- and 12.5 μg/ml-induced apoptosis. The higher dose of *Lcr*S_{5-30} induced a significant amount of apoptosis in THP-1 cells, but this was inefficiently abrogated by SB431542. Thus, *Lcr*S_{5-30} could trigger TGF-β-independent apoptosis.

In conclusion, probiotic *Lcr* produces heat-stable molecules with an MW range of 5–30 kDa, primarily proteins, which promoted lymphocyte, monocyte and THP-1 cell apoptosis without affecting intestinal epithelial cells. *Lcr*S_{5-30} triggered apoptosis of immune cell *in vitro* by a mitochondrial pathway, but not via the TGF-β signaling pathway. *Lcr*S_{5-30} also inhibited LPS-induced inflammatory cytokines in activated immune cells. This investigation demonstrated a role for *Lcr*S_{5-30} in promoting apoptosis of immune cells and suggests the possibility of a probiotics-based regimen for prevention of IBD.

Conflict of interest

The authors declare that they have no competing interests.

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