

# Identification of Immunopotentiating Lactic Acid Bacteria that Induce Antibody Production by *in vitro* Stimulated Human Peripheral Blood Mononuclear Cells

Makiko YAMASHITA<sup>1</sup>, Akira HITAKA<sup>2</sup>, Himiko FUJINO<sup>2</sup>, Takashi MATSUMOTO<sup>3</sup>,  
Takanori HASEGAWA<sup>3</sup>, Fumiki MORIMATSU<sup>3</sup>, Tsukasa FUJIKI<sup>1</sup> and Yoshinori KATAKURA<sup>1,2,\*</sup>

<sup>1</sup>Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

<sup>2</sup>Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

<sup>3</sup>R&D Center, Nippon Meat Packers, 3–3 Midorigahara, Tsukuba, Ibaraki 300-2646, Japan

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L-leucyl-L-leucine methyl ester (LLME) is known to remove lysosome-rich cells from human peripheral blood mononuclear cells (PBMCs). To evaluate the immunopotentiating ability of lactic acid bacteria (LAB), we adopted the *in vitro* stimulation protocol of LLME-treated PBMCs as a model assay system and monitored the level of antibody produced by stimulated PBMCs. The results indicated that several LAB strains have immunopotentiating ability against PBMCs, as evidenced by the enhanced antibody production and increased number of antigen-specific B cells. Next, we identified T cells as the direct target cells of the immunopotentiating LAB strain L32, suggesting that L32 induced antibody production by PBMCs through T-cell activation. Finally, we tested the immunopotentiating ability of ligands for Toll-like receptor 2 (TLR2), which is known to mediate the LAB signal, and observed that both L32 and one of the TLR2 ligands, LTA-BS, induced antigen-specific antibody production by *in vitro* stimulated PBMC. This suggests that L32 and LTA-BS can be used as an adjuvant for stimulating immune reaction in PBMCs.

**Key words:** lactic acid bacteria; immunopotentiating ability; human peripheral blood mononuclear cells; Toll-like receptor

## INTRODUCTION

Lactic acid bacteria (LAB) are known to have various beneficial effects [1]. Some of these effects are elicited through modulation of the function of immune cells, including intestinal epithelial cells (IECs), M cells, dendritic cells (DCs) and T cells [2]. For example, some LAB strains can directly act on IEC to maintain the epithelial barrier integrity [3] and activate macrophages and DC depending on Toll-like receptors to produce several cytokines in order to modulate immune responses [4, 5]. In addition, LAB strains increase and activate natural killer cells and neutrophils, which have anti-infectious and anticancer abilities [6]. Recently, researchers have explored the capacity of LAB to induce the development of Treg cells, which are critically involved in controlling immunopathology in a wide

variety of inflammatory diseases, and reported that some LAB strains elicit Treg cell development through DC and IEC involvement [4, 7].

In this study, we focused on immunopotentiating LAB strains. We adopted the *in vitro* stimulation system of human peripheral blood mononuclear cells (PBMCs) to screen for immunopotentiating LAB strains [8]. In this system, PBMCs are first treated with L-leucyl-L-leucine methyl ester (LLME) to remove lysosome-rich cells, including monocytes and NK cells, thus resulting in PBMCs that mainly comprise T and B cells. We attempted to identify immunopotentiating LAB strains that induce immunoglobulin production by LLME-treated PBMCs through direct T- or B-cell activation.

## MATERIALS AND METHODS

### Preparation of bacteria

*Lactobacillus casei* (L25), *Streptococcus thermophilus* (L26), *L. acidophilus* (L27), *L. lactis* (L28), *L. paracasei* (L31), *L. plantarum* (L32), *L.*

\*Corresponding author. Mailing address: Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Phone & Fax: +81-92-642-3050. E-mail: katakura@grt.kyushu-u.ac.jp

*gasseri* (L33), and *L. salivarius* subsp. *salivarius* (L34) were washed twice and freeze-dried after heating at 100°C for 30 min. LAB were then suspended in  $2.24 \times 10^{-2}$  M phosphate buffer containing  $1.37 \times 10^{-1}$  M NaCl (PBS). The final working concentration in medium was adjusted to 1–10 µg/mL.

#### Isolation of PBMCs

PBMCs were isolated by density gradient centrifugation using the lymphocyte separation medium (LSM; Organon Teknika, Durham, NC, USA). In brief, 25 mL of peripheral blood was layered onto 15 mL of LSM and centrifuged at  $400 \times g$  for 30 min at room temperature. PBMCs were collected, washed thrice with eRDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) and treated with 0.25 mM LLME (Bachem, Torrance, CA, USA) for 20 min at room temperature [8]. The cells were used for further analyses after washing with culture medium.

All experiments in this study were carried out in accordance with the Declaration of Helsinki and the ethics committee regulations of the Faculty of Agriculture, Kyushu University.

#### Bacterial coculture and PBMC stimulation

*In vitro* stimulation of human PBMCs was performed in 24-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) as described previously [8]. LLME-treated PBMCs ( $5 \times 10^6$  cells) were sensitized with  $\beta$ -lactoglobulin (10 µg/mL; Wako, Osaka, Japan) in the presence of IL-2 (10 units/mL; R&D Systems, Minneapolis, MN, USA), IL-4 (10 ng/mL; PeproTech, London, UK) and CpG2006 oligodeoxynucleotide (CpG2006 ODN, 52-TCGTCGTTTTGTCGTTTTGTCGT-32, 1 µg/mL; Sigma-Genosys, Ishikari, Japan) or LAB (10 µg/mL) and cultured in eRDF medium supplemented with 10% heat-inactivated FBS and 2-ME (50 µM) for 7 days.

#### Cell isolation

T and B cells were isolated using a MACS system (Miltenyi Biotec K.K., Tokyo, Japan). T cells were isolated using CD3 MicroBeads according to the positive selection protocol. B cells were isolated using the a B-cell Isolation Kit according to the manufacturer's protocol. The purity of the isolated cells was checked by a flow cytometer (EPICS XL, Beckman Coulter, Miami, FL, USA) using APC-labeled anti-CD3 and FITC-labeled anti-CD19 antibodies (Beckman Coulter).

#### ELISA

Microtiter plates (Nunc, Naperville, IL, USA) were

coated with an anti-human IgM, anti-human IgG or anti-human IgA antibody (TAGO, Burlingame, CA, USA) diluted with 0.1 M sodium carbonate buffer (pH 9.6) and incubated for 2 hr at 37°C. After washing the plates thrice with PBS containing 0.05% Tween 20 (TPBS), aliquots of serially diluted supernatants of *in vitro* stimulated PBMCs were added to the plate and incubated overnight at 4°C. After washing thrice with TPBS, the diluted horseradish peroxidase-conjugated anti-human IgM, anti-human IgG or anti-human IgA goat antibody (TAGO) were added and subsequently incubated for 2 hr at 37°C. After washing thrice with TPBS, a substrate solution [0.1 M citrate buffer (pH 4.0) containing 0.003% H<sub>2</sub>O<sub>2</sub> and 0.3 mg/mL ABTS (Wako)] was added and incubated for 20 min. Absorbance at 405 nm was measured using an ELISA reader.

#### ELISPOT assay

A MultiScreen 96-well plate (Millipore, Bedford, MA, USA) was coated with 100 µg/mL  $\beta$ -lactoglobulin in 0.1 M sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing thrice with PBS, the plate was blocked with 200 µL of 1% fish gelatin in PBS for 3 hr at 37°C. The plate was then washed thrice with PBS, following which 100 µL of the single-cell suspension ( $1 \times 10^5$  cells/well) was seeded and incubated overnight at 37°C. After washing five times with TPBS, 100 µL of diluted horseradish peroxidase-conjugated anti-human IgM goat antibody was added and subsequently incubated for 2 hr at 37°C. After washing five times with TPBS, 50 µL of TrueBlue (KPL, Gaithersburg, MD, USA) was added to the plate and incubated at room temperature for 10 min. The plate was then washed with water and dried. The number of spots was counted using the ImageJ software.

#### Immunofluorescence staining and flow cytometric analysis

Cells were stained with fluorescence-labeled mouse anti-human mAb, which included FITC/PE-conjugated anti-CD4, FITC-labeled anti-CD19, APC-labeled anti-CD38, ECD-labeled anti-CD69, PE-labeled anti-CD80 and PE-labeled anti-CD86 mAbs (Beckman Coulter). Analyses were performed with a flow cytometer (EPICS XL) and the FlowJo software (Tree Star, San Carlos, CA, USA). Phenotypical results were expressed as the percentage of positive cells with respect to the total number of gated lymphocytes or a particular cell subset.

#### Toll-like receptor ligands

We used Toll-like receptor ligands, such as

lipoteichoic acid from *Bacillus subtilis* (LTA-BS, TLR2 agonist), Pam3CSK4 (TLR2/TLR1 agonist), FSL-I (synthetic diacylated lipoprotein, TLR2/TLR6 agonist), and CpG2006 (TLR9 agonist) (InvivoGen, San Diego, CA, USA), to stimulate PBMCs.

## RESULTS AND DISCUSSION

### Screening for immunopotentiating lactic acid bacteria

To evaluate the immunopotentiating ability of lactic acid bacteria (LAB) against human PBMCs, we used the *in vitro* stimulation protocol of human PBMCs as a model assay system and monitored the level of antibody produced by stimulated PBMCs. Human PBMCs were first treated with LLME to remove lysosome-rich cells, including monocytes and NK cells, and subsequently stimulated with  $\beta$ -lactoglobulin in the presence of IL-2, IL-4, and LAB or CpG2006 ODN. After 7 days of culture, the amount of total antibody and antigen-specific antibody produced was evaluated by ELISA and ELISPOT assays, respectively (Fig. 1 and Fig. 2). Several LAB strains as well as CpG2006 ODN were found to induce antibody production by PBMCs. Interestingly, LAB strains induced IgG production, whereas CpG2006 ODN stimulated IgA and IgM production by PBMCs (Fig. 1). Furthermore, both LAB strains and CpG2006 ODN increased the number of B cells producing antigen-specific antibody (Fig. 2), indicating that these LAB strains and CpG2006 ODN have strong immunopotentiating abilities against human PBMCs and that the *in vitro* stimulation protocol of PBMCs is useful for immunopotentiating LAB screening. In addition, we observed a close relationship between IgM production and antigen-specific IgM-producing cell induction (Fig. 1 and 2), indicating that L31 to L34 LAB strains induce IgM production and antigen-specific IgM-producing cells in *in vitro* stimulation of PBMCs.

### Identification of target cells of immunopotentiating LAB strains

For further analyses, we focused on the LAB strain *L. plantarum* L32. First, we evaluated the activation status of T and B cells in PBMCs stimulated *in vitro* in the presence of CpG2006 ODN or L32. The results clearly indicated that T cells (CD4<sup>+</sup> and CD4) and B cells in PBMCs stimulated *in vitro* were both activated by the addition of CpG2006 ODN and L32 as compared with the control, in which CD69 was used as the activation marker for T cells and CD80 and CD86 were used as activation markers for B cells, respectively (Fig. 3). This also indicated that L32 and CpG2006 ODN activated

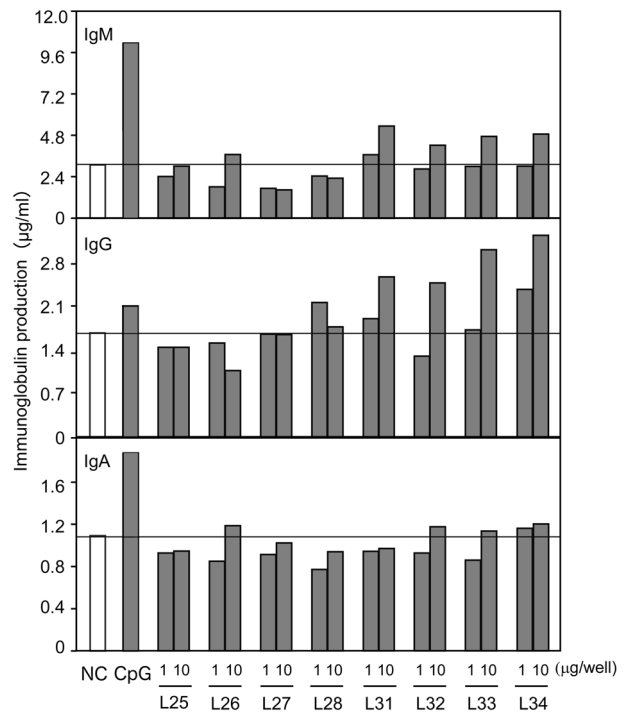


Fig. 1. The effects of LAB on total antibody production by human PBMCs stimulated *in vitro*. LLME-treated PBMCs were stimulated *in vitro* with  $\beta$ -lactoglobulin in the presence of IL-2, IL-4 and LAB or CpG2006 ODN. After 7 days of culture, the amount of IgM, IgG, and IgA antibodies produced were evaluated by ELISA.

both T and B cells during the course of *in vitro* stimulation of PBMCs.

Next, we attempted to identify the direct target cells of L32 LAB and CpG2006 ODN. CD3<sup>+</sup> T cells were isolated from PBMCs and stimulated *in vitro* in the presence of CpG2006 ODN or L32. Subsequently, CD69 expression in T cells was monitored by a flow cytometer (Fig. 4A). We observed that T cells stimulated *in vitro* in the presence of CpG2006 ODN or L32 were activated, indicating that CpG2006 ODN and L32 activated CD3<sup>+</sup> T cells on *in vitro* stimulation of PBMCs. Furthermore, CD19<sup>+</sup> B cells were isolated from PBMCs and stimulated as described above, and CD86 and CD38 expression in the B cells was monitored by a flow cytometer (Fig. 4B). CpG2006 ODN increased the number of CD86<sup>+</sup> and CD38<sup>+</sup> cells, indicating that CpG2006 ODN directly activated B cells on *in vitro* stimulation of PBMCs. Conversely, L32 did not increase the number of activated B cells, thereby suggesting that CpG2006 ODN and L32 exhibit immunopotentiating ability against PBMCs via different mechanisms and that L32 induces antibody production by PBMCs through the

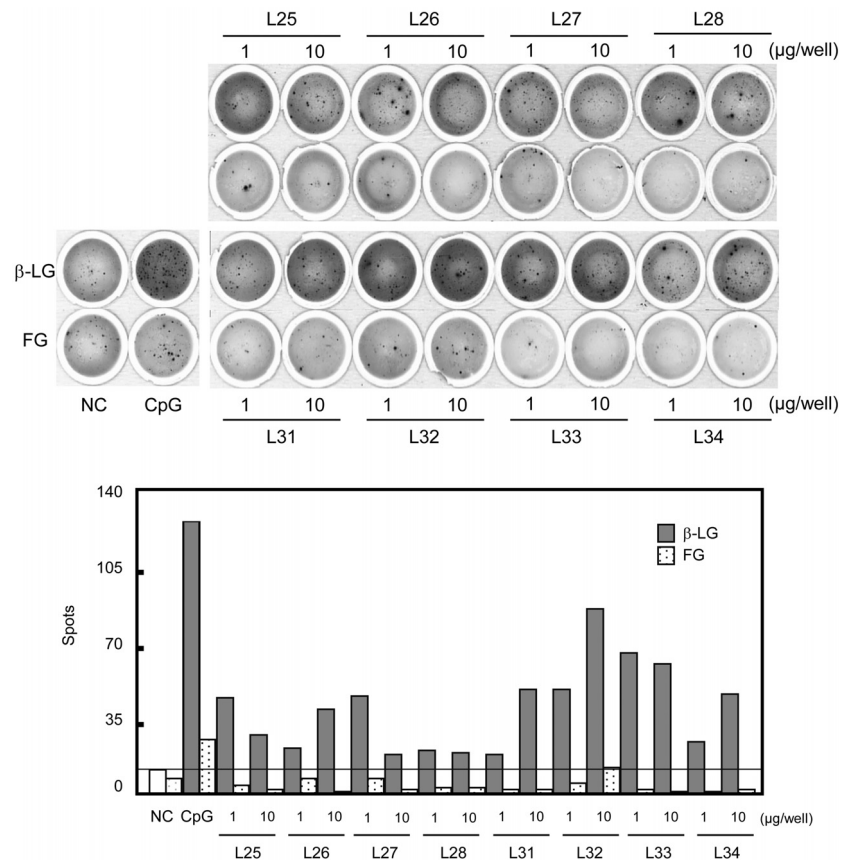


Fig. 2. The effects of LAB on antigen-specific antibody production by human PBMCs stimulated *in vitro*. LLME-treated PBMCs were stimulated *in vitro* with  $\beta$ -lactoglobulin in the presence of IL-2, IL-4, and LAB or CpG2006 ODN. After 7 days of culture, B cells producing antigen-specific antibody were detected by an ELISPOT assay. Wells were coated with  $\beta$ -lactoglobulin for detecting antigen-specific antibody-producing cells or with fish gelatin (FG) for non-specific antibody-producing cells. The number of spots in A were counted using the ImageJ software and depicted in B. NC, negative control.

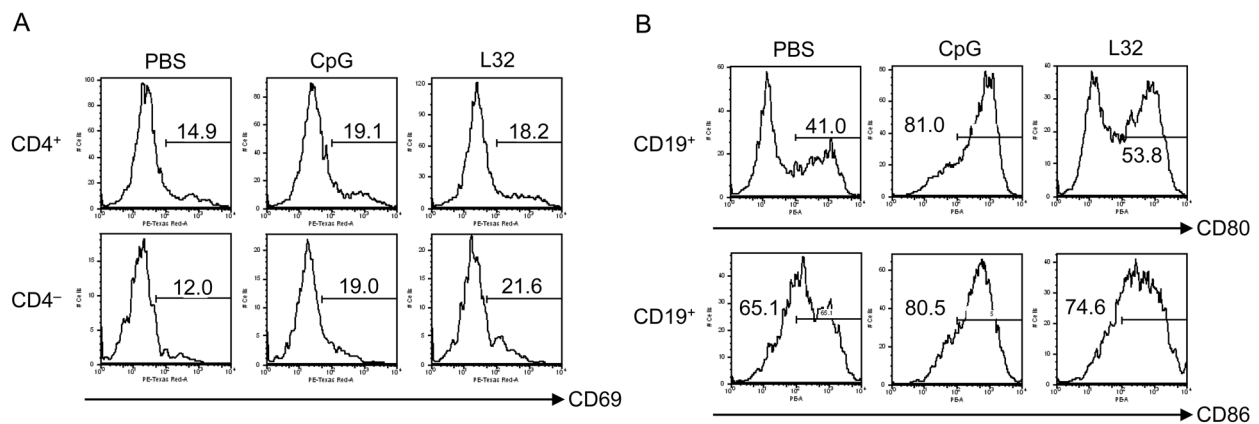


Fig. 3. Activation profile of PBMCs stimulated *in vitro* in the presence of CpG2006 ODN or L32. A: After LLME-treated PBMCs were stimulated *in vitro* as described above, CD3<sup>+</sup> T cells were isolated, and CD69 expression in CD4<sup>+</sup>/CD4<sup>-</sup> T cells was monitored by a flow cytometer. B: After LLME-treated PBMC were stimulated *in vitro* as described above, CD19<sup>+</sup> B cells were isolated, and CD80 and CD86 expression was monitored by a flow cytometer.

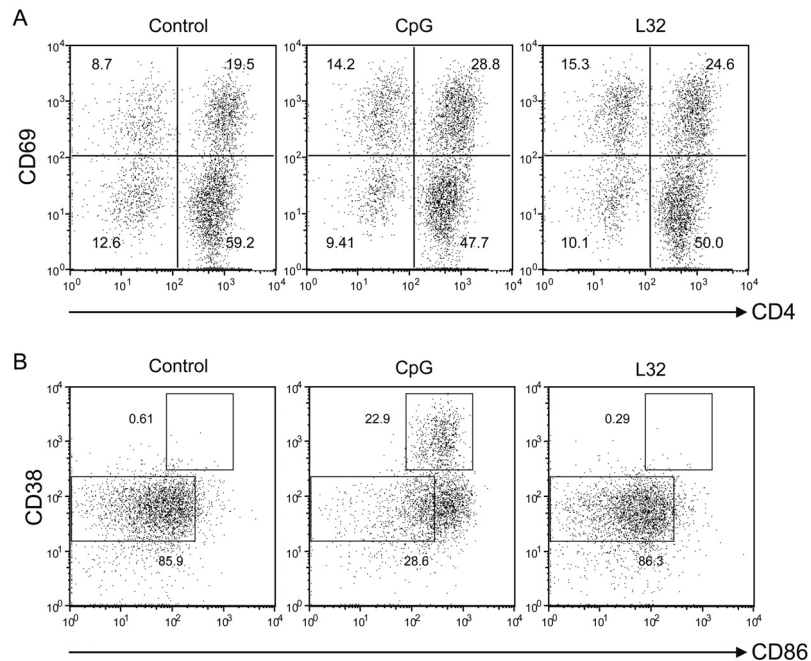


Fig. 4. Direct target cells of LAB at *in vitro* stimulation of PBMCs. A: CD3<sup>+</sup> T cells were isolated from LLME-treated PBMCs and stimulated *in vitro* as described above. The relative number of CD69<sup>+</sup> cells was monitored by a flow cytometer. B: CD19<sup>+</sup> B cells were isolated from LLME-treated PBMCs and stimulated *in vitro* as described above. The relative number of CD86<sup>+</sup> and CD38<sup>+</sup> cells was monitored by a flow cytometer.

activation of T cells.

#### *Toll-like receptor ligands induce antibody production by PBMCs*

Immune cells express pattern-recognition receptors, such as TLRs, that are specific to various microbial components. Several LAB strains are known to regulate and stimulate immune cell functions in a TLR2-dependent manner [2]. In this study, we investigated whether TLR2 ligands, rather than LAB strains, stimulate PBMCs and induce their antibody production. The TLR2 ligands used in this study include LTA-BS specific to TLR2, Pam3CSK4 specific to TLR2/TLR1 and FSL-I specific to TLR2/TLR6. T and B cells were isolated from PBMCs and stimulated *in vitro* with an antigen in the presence of the TLR ligand. The results demonstrated that all these TLR ligands activated T cells in contrast to the control (Fig. 4A and B, Fig. 5A and B). Furthermore, Pam3CSK4 and FSL-I, but not LTA-BS, increased the number of CD86<sup>+</sup> cells. The activation profiles of T and B cells with LTA-BS were similar to those with L32, suggesting that LTA-BS would substitute for an L32 signal. To confirm this, antigen-

specific antibody production by PBMCs stimulated in the presence of TLR ligands was evaluated by the ELISPOT assay (Fig. 5C). Among the TLR2 ligands tested, LTA-BS markedly increased B cells that produce antigen-specific antibody from *in vitro* stimulated PBMCs. Although the reason why LTA-BS, but not Pam3CSK4 and FSL-I, induced the antigen-specific B cells by *in vitro* stimulation of PBMCs remains unknown, L32 would induce antibody production through any other mechanisms besides T-cell activation. However, these results suggest that the common structure of *L. plantarum* L32 and LTA-BS explains their ability to stimulate PBMCs and that *L. plantarum* L32 stimulates T cells via TLR2, thereby activating antigen-specific immune reaction in PBMCs.

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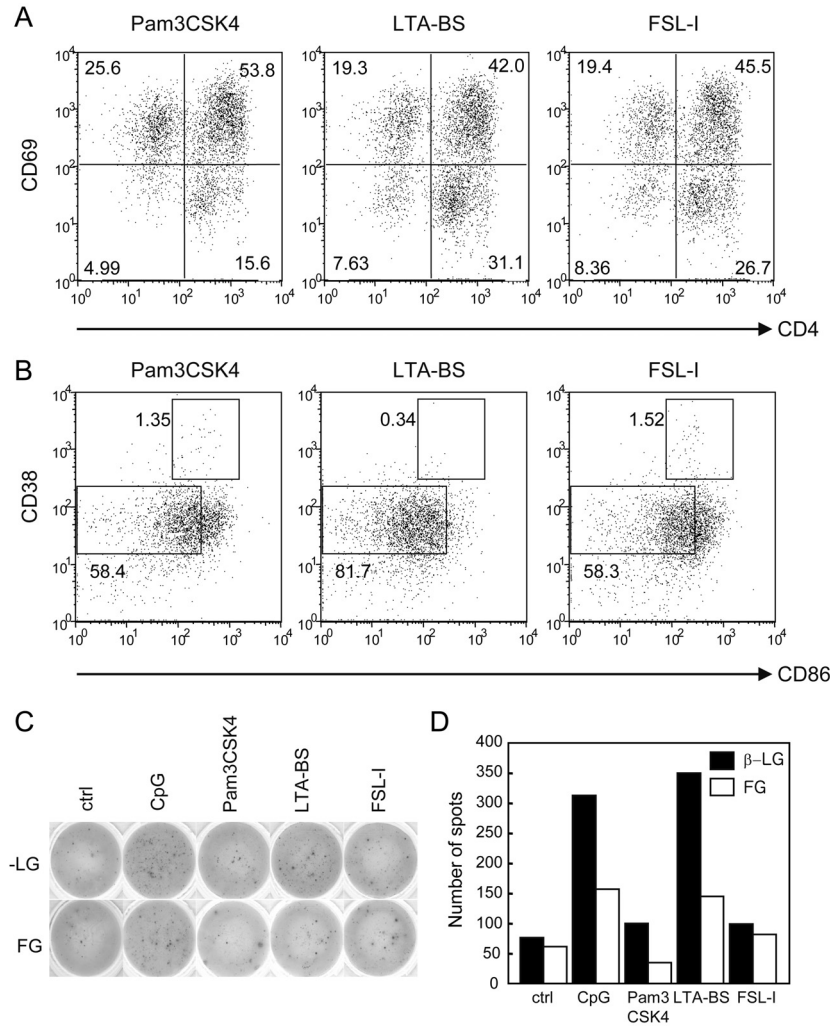


Fig. 5. TLR2 ligands induce antibody production by PBMCs. A: CD3<sup>+</sup> T cells were isolated from the LLME-treated PBMCs and stimulated *in vitro* as described above in the presence of TLR2 ligands (Pam3CSK4, LTA-BS and FSL-I). The relative number of CD69<sup>+</sup> cells was monitored by a flow cytometer. B: CD19<sup>+</sup> B cells were isolated from LLME-treated PBMCs and stimulated *in vitro* as described above in the presence of TLR2 ligands. The relative number of CD86<sup>+</sup> and CD38<sup>+</sup> cells were monitored by a flow cytometer. C: LLME-treated PBMCs were stimulated *in vitro* as described above in the presence of TLR2 ligands. After 7 days of culture, B cells producing antigen-specific antibody were detected by an ELISPOT assay as described above. The number of spots in C were counted using the ImageJ software and depicted in D.

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