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Bovine β-Casein (1−28), a Casein Phosphopeptide, Enhances Proliferation and IL-6 Expression of Mouse CD19$^+$ Cells via Toll-like Receptor 4

KEISUKE TOBITA, TAKESHI KAWAHARA,* AND HAJIME OTANI

Integrated Department of Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan

This study was conducted to elucidate the target cells and receptors which participate in the mitogenic and interleukin (IL)-6-enhancing effect of bovine β-casein (1−28), a casein phosphopeptide. When the spleen lymphocyte subset (CD4$^+$, CD8$^+$, and CD19$^+$ cells) from C3H/HeN mice was cultured with the β-casein (1−28), it exerted a dose-dependent mitogenic effect on CD19$^+$ cells. The effect of β-casein (1−28) was not apparent in the case of CD19$^+$ cells from C3H/HeJ mouse. In addition, the effect was significantly inhibited by treating the C3H/HeN mouse-derived CD19$^+$ cells with neutralizing antibody for toll-like receptor 4 (TLR4). Reverse transcription-polymerase chain reaction analysis showed that the β-casein (1−28) exerted an IL-6-enhancing effect on the CD19$^+$ cells. The effect was also abrogated in either C3H/HeJ mouse-derived CD19$^+$ cell culture or the anti-TLR4 antibody-added culture. These results suggest that the β-casein (1−28) stimulates both proliferation and IL-6 expression of CD19$^+$ cells via TLR4.

KEYWORDS: Casein phosphopeptide; milk; mitogenic activity; CD19$^+$ cells; IL-6; toll-like receptor 4

INTRODUCTION

Bioactive peptides generated from food constituent proteins by gastrointestinal enzymatic digestion have drawn attention in recent years. Mammalian milk, in particular bovine milk, is a typical protein-rich food and used as a versatile foodstuff. Many researchers, therefore, have characterized milk protein derived functional peptides (*1*−*3*).

Casein phosphopeptides (CPP) are phosphorylated peptides resulting from the proteolysis of calcium-sensitive caseins. They possess a phosphoserine (SerP)-rich region, consisting of SerP residues, such as αs1-casein Glu^{63}-SerP-Ile-SerP-SerP-SerP-Glu-Glu^{70} or β-casein Glu^{14}-SerP-Leu-SerP-SerP-SerP-Glu-Glu^{21}. The property of CPP allows them to form complexes with calcium ions or other mineral ions (*4*). For this reason, the effect of CPP in preventing precipitates and increasing absorption of calcium in the intestine has been demonstrated in both *in vitro* and *in vivo* studies (*5*, *6*). The usage of CPP has become widespread to promote remineralization for preventing osteoporosis (*7*), anemia (*8*), and caries (*9*).

However, our recent study substantiated that some CPP had immunoenhancing activities. Hata et al. (*10*) found that CPP-III, a commercially available CPP preparation mainly consisting of β-casein (1−28) and αs2-casein (1−32) (*11*), stimulated proliferation by mouse spleen cells. CPP-III was also shown to enhance intestinal IgA levels by promoting the production of IL-6 using oral administration of the CPP-III-supplemented diet in mice (*12*). In parallel with these studies, β-casein (1−28), a major component of CPP-III, was found to have both mitogenic and immunoglobulin-enhancing effects in mouse spleen cell cultures (*13*). The critical role of β-casein (1−28) in IgA production has become clear (*14*), and the sequence SerP-X-SerP was revealed to be essential for these immunoenhancing activities of β-casein (1−28) by using chemically synthesized partial fragments of the β-casein (1−28) (*15*). However, little is known about what subset of lymphocytes interacts with β-casein (1−28) predominantly, and what receptor is involved in the β-casein (1−28)-induced immunoenhancing activity.

Thus, in this paper, we sought to elucidate the lymphocyte subset and receptor involving the immunoenhancing activities of β-casein (1−28).

MATERIALS AND METHODS

Reagents. CPP-III was provided by Meiji Seika (Tokyo, Japan). Lipopolysaccharide (LPS) from *Salmonella typhimurium* was purchased from Sigma Chemical (St. Louis, MO).

Preparation of β-Casein (1−28) from CPP-III. Bovine β-casein (1−28) was purified from CPP-III with an anion-exchange chromatography technique partially according to our previous paper (*15*). The peptide purified by a DEAE Sephadex A-50 column chromatography (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was identified as β-casein (1−28) by comparing its retention time on reverse-phase high-performance liquid chromatography (RP-HPLC) with that of standard β-casein (1−28). The standard was investigated for its amino acid sequences using a protein sequencer model 476A (Applied Biosystems, Foster City, CA) and time-of-flight mass spectrometer (*15*). The RP-HPLC was carried out with a Shimadzu System 10 instrument

* Author to whom correspondence should be addressed. Fax: +81-265-77-1431. E-mail: tkawafb@shinshu-u.ac.jp.

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(Shimadzu, Kyoto, Japan) equipped with a TSK-Gel ODS 80 TM column (0.6 × 15 cm, Tosoh, Tokyo, Japan). The separating conditions of the RP-HPLC were determined as follows: linear gradient of a mixture of filtrated distilled water and acetonitrile including 0.05% (v/v) trifluoroacetic acid at 5−40% acetonitrile for 10 min at a flow rate of 0.8 mL/min. Elution of the effluent was detected by monitoring its absorbance at 214 nm. The prepared β-casein (1−28)-containing solution was checked with Limulus Amebocyte Lysate (LAL) test kit (Cape Cod, Falmouth, MA). Gelatinization of the β-casein (1−28)-containing solution after mixing the LAL solution was according to the manufacturer's recommendations.

Mice. Specific pathogen-free male C3H/HeN and C3H/HeJ mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and housed at 23 °C ± 3 °C with 12 h−12 h light−dark cycle. Mice at 6 weeks of age were used in accordance with the Guideline for Regulation of Animal Experimentation in the Faculty of Agriculture of Shinshu University.

Preparation and Culture of Spleen Cells. Mice were killed by vertebral dislocation, and their spleens were aseptically removed. Single-cell suspension was prepared by gentle manipulation of the spleen tissues in RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan). The cells were washed twice with the medium and resuspended at a concentration of 6×10^6 viable cells/mL in the medium containing 5% fetal bovine serum (HyClone, Logan, UT), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cell culture was set up in quadruplicate on flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA). Each well was filled with 100 μL of the cell suspension and 20 μL of the β-casein (1−28) or LPS solution dissolved in 0.01 M phosphate-buffered saline at pH 7.2 containing 0.15 M NaCl. The final concentrations were spleen cells, 5×10^6 viable cells/mL; β-casein (1−28), 0−500 μg/mL; and LPS, 5×10^{-1} μg/mL. The mixture was cultured in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Proliferation of cells was estimated by counting the cells using a hematocytometer after staining with trypan blue. To prepare $CD4^+$, $CD8^+$, and $CD19^+$ cell subsets from the spleen cells, a pooled cell suspension from mice (three to four mice for $CD4^+$, six to seven mice for $CD8^+$, and two mice for $CD19^+$) was subjected to a Magnetic Cell Sorting system (MACS Miltenyi Biotec GmbH, Germany) according to the supplier's recommendations. To reveal the involvement of toll-like receptor (TLR), anti-mouse TLR2 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse TLR4 rabbit antibody (Santa Cruz) was added to culture 1 h prior to treatment with β-casein (1−28) or LPS according to the method of Hana et al. (*16*). Contamination with exogenous LPS was ruled out by assessing the effect of β-casein (1−28) in the presence of polymyxin B (PMB, Nacalai Tesque, Kyoto, Japan). After mixing the β-casein (1−28) or LPS with the PMB for 30 min, the effects of these samples on the proliferation of the $CD19^+$ cell culture were evaluated.

Preparation of Total RNA and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR). Total RNA from $CD19^+$ cells were extracted with the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). The reaction for RT was carried out by adding 1 mM each of dNTP, 2.5 units/μL M-MLV reverse transcriptase (Invitrogen), and 10 pmol/μL oligo $d(T)_{18}$ primer to 1 μg of total RNA, before incubating at 42 °C for 50 min. The resulting complementary DNA was subjected to PCR with a *Taq* PCR Core Kit (Qiagen, Chatsworth, CA) and 10 pmol/μL primers for IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for amplifying the cytokines and GAPDH were as follows: IL-6 (sense), 5′-CTG GTG ACA ACC ACG GCC TTC CCT A-3′; IL-6 (antisense), 5′-ATG CTT AGG CAT AAC GCA CTA GGT T-3′ (*17*); GAPDH (sense), CCA CAG TCC ATG CCA TCA CTG CC-3′; and GAPDH (antisense), GGT CCA CCA CCC TGT TGC TGT AG-3′ (from base 567−589 and 1019−997, respectively, accession number M32599). PCR was run for 30 cycles of denaturation (94 °C, 1 min), primer annealing (60 °C, 1 min), and extension (72 °C, 1 min) with a PTC-150 MiniCycler (MJ Research, Waltham, MA). Amplified DNA was electrophoresed on 2% agarose gel in 0.04 M tris-acetate buffer (pH 8.0) containing 1 mM EDTA, and then visualized by ethidium bromide staining. The fluorescent intensities of the bands were digitalized with a Printgraph (Atto, Tokyo, Japan), and quantified with Densitograph Lane Analyzer software (Atto). Data were normalized to the expression of GAPDH.

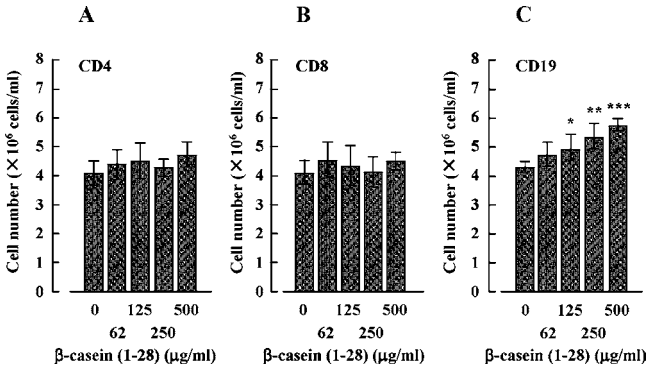

Figure 1. Effect of β-casein (1–28) on proliferative responses of mouse spleen lymphocyte subsets. $CD4^+$ Cells (**A**), $CD8^+$ Cells (**B**), and $CD19^+$ cells (**C**) were stimulated with 0, 62, 125, 250, and 500 μg/mL β-casein (1–28) for 72 h. These data represent means ± SD ($n = 4$). The value was significantly different from that under β-casein (1–28)-free condition at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in two-sided Student's *t* test.

Statistical Analysis. Results were expressed as means ± standard deviation for evaluating the cell proliferation. The significance of the difference between the β-casein (1−28)-free culture and β-casein (1−28)-added culture was tested by two-sided Student's *t* test.

RESULTS

$CD19^+$ Cells Are Responsive to Stimulation with β-Casein (1−28). We primarily assessed the lymphocyte subsets responsive to stimulation with β-casein (1−28). Proliferation of CD4, CD8, and $CD19^+$ cell subsets from C3H/HeN mouse after 72 h of culture is shown in **Figure 1**. In the presence of 125−500 μg/mL β-casein (1−28), $CD19^+$ cells were significantly increased in a dose-dependent manner, and reached 1.31-fold compared with the β-casein (1−28)-free condition (**Figure 1A**). After the experimental period, no dramatic effect on the survival rate of the cells was observed in any cell culture. Although the proliferation of the $CD4^+$ cells also tended to be enhanced by β-casein (1−28), it was insignificant at any concentration tested (**Figure 1B**). In addition, β-casein (1−28) tested had no effect on the proliferation of $CD8^+$ cells (**Figure 1C**).

TLR4-Deficit Mouse-Derived $CD19^+$ Cells Were Irresponsive to Stimulation with β-Casein (1−28). The effect of β-casein (1−28) on proliferation of $CD19^+$ cells from C3H/HeN and C3H/HeJ mice was investigated. After 72 h of culture, not less than 125 μg/mL of β-casein (1−28) significantly enhanced the proliferation of $CD19^+$ cells from C3H/HeN mouse (**Figure 2A**). In contrast, no significant effect was apparent even when the C3H/HeJ mouse-derived $CD19^+$ cells were cultured with 500 μg/mL of β-casein (1−28) (**Figure 2B**).

Anti-TLR4 Antibody Inhibited β-Casein (1−28)-Induced Proliferation. To elucidate the involvement of TLR4 in the mitogenic effect of β-casein (1−28), the effect of the presence of anti-TLR4 antibody was investigated. As shown in **Figure 3C**, the anti-TLR4 antibody significantly inhibited the proliferation of cells by 250 μg/mL β-casein (1−28), while the proliferation was not influenced under the antibody-free (**Figure 3A**) or the anti-TLR2 antibody-added conditions (**Figure 3B**).

Moreover, to assess whether or not the TLR4-mediated mitogenic effect of β-casein (1−28) was due to the LPS, the effect after treatment with PMB was investigated. Treatment with the 2 μg/mL PMB against the 62−250 μg/mL β-casein (1−28) showed no significant effect (**Figure 4A**) in contrast to the LPS (**Figure 4B**).

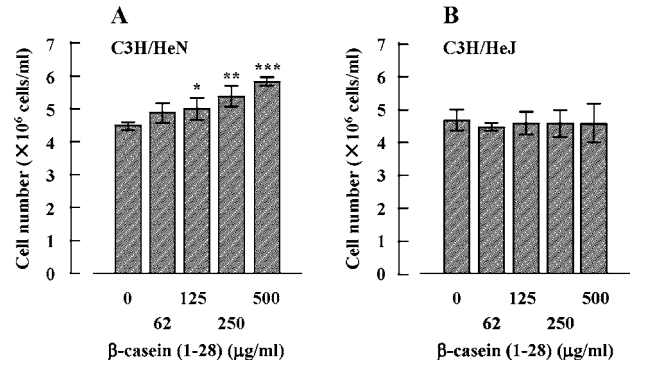

Figure 2. Effect of β-casein (1–28) on proliferative responses and cytokine expressions of CD19+ cells from C3H/HeN and C3H/HeJ mouse. Cells from C3H/HeN mice (**A**) and C3H/HeJ mice (**B**) were stimulated with 0, 62, 125, 250, and 500 μg/mL β-casein (1–28) for 72 h. These data represent means ± SD ($n = 4$). The value was significantly different from that under β-casein (1–28)-free condition at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in two-sided Student's t test.

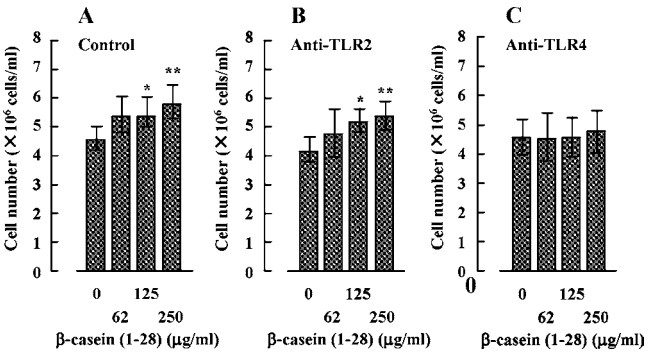

Figure 3. Effect of β-casein (1–28) on proliferative responses of CD19+ cells in the presence of anti-TLR antibody. CD19+ cells from C3H/HeN mice were stimulated with 0, 62, 125, 250, and 500 μg/mL β-casein (1–28) for 72 h in the absence (**A**) or presence of 2.5 μg/mL anti-TLR2 (**B**) and -TLR4 (**C**) antibody. These data represent means ± SD ($n = 4$).The value was significantly different from that under β-casein (1–28)-free condition at $^{*}P < 0.05$, and $^{**}P < 0.01$ in two-sided Student's t test.

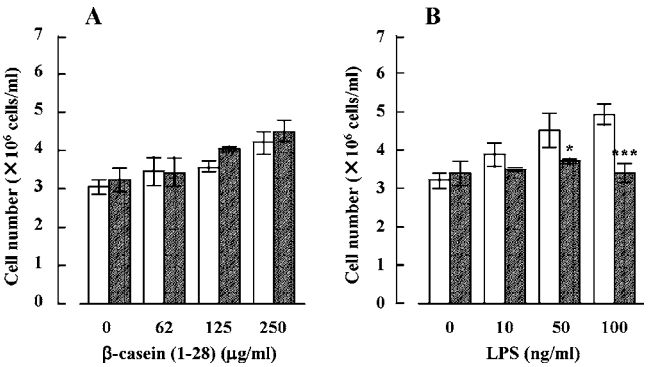

Figure 4. Effect of β-casein (1–28) on proliferative responses of CD19+ cells in the presence of PMB. CD19+ cells from C3H/HeN mice were stimulated with 0, 62, 125, and 250 μg/mL β-casein (1–28) (**A**) or 10, 50, and 100 ng/mL LPS (**B**) for 72 h in the absence (open bar) or presence (shaded bar) of 50 μg/mL PMB. These data represent means ± SD ($n = 4$). The value was significantly different from that under PMB-free condition at $^{*}P < 0.05$, and $^{***}P < 0.001$ in two-sided Student's t test.

β-Casein (1−28) Enhanced Expression of IL-6 by CD19+ Cells via TLR4-Mediated Signal Pathway. We investigated whether or not the mRNA expression for IL-6, one of the cytokines induced by TLR4-mediated signaling, was affected by β-casein (1−28). Time course of the expression is shown in **Figure 5A**. RT-PCR analysis revealed that the expression of IL-6 increased gradually with time approximately up to 2.7-fold compared with that of the β-casein (1−28)-free condition. In addition, **Figure 5B** shows that the CD19+ cells from C3H/HeJ mouse showed impaired expression of IL-6 by stimulation with β-casein (1−28). This result suggests the hypothesis that β-casein (1−28) interacts with TLR4 to induce the expression of IL-6. Thus, we further examined the involvement of TLR4 on the expression of IL-6 by β-casein (1−28) using the anti-TLR4 antibody. As shown in **Figure 5C**, the anti-TLR4 antibody critically abrogated the IL-6 expression. Furthermore, in the presence of PMB, the expression of IL-6 by 500 μg/mL of β-casein (1−28) was not inhibited compared with 0.5 μg/mL of LPS which can induce expression of IL-6 at the same level as 500 μg/mL of β-casein (**Figure 5D**).

DISCUSSION

Proliferation-enhancing substances called mitogen are known to be recognized by specific receptors and trigger the activation of naive lymphocytes without prior sensitization. Today, it is widely accepted that many mitogenic actions by bacteria-derived substances result from TLR-family-mediated signal transduction. For example, peptidoglycan and LPS are known as ligands for TLR2 (*18*) and TLR4 (*19*), respectively.

Among the CD4+, CD8+, and CD19+ cell subsets, the β-casein (1−28) exerted a significant mitogenic effect on CD19+ cells from C3H/HeN mice (**Figure 1**). In contrast, in the proliferation of CD19+ cells from C3H/HeJ mice, the β-casein (1−28) was shown to have little effect (**Figure 2**). C3H/HeJ mice are hyporesponsive to LPS because of the mutation in the exon of the TLR4 gene (*20*). The defective response of CD19+ cells from C3H/HeJ mouse therefore indicates that β-casein (1−28) requires TLR4 to exhibit a mitogenic effect.

Moreover, **Figure 3** shows that the effect of β-casein (1−28) on CD19+ cells from C3H/HeN mice was significantly inhibited in the presence of the TLR4-neutralizing antibody. These results strengthen the hypothesis that β-casein (1−28) induces proliferation via TLR4.

Previous studies have revealed that a variety of non-LPS ligands such as fibronectin (*21*) and heat shock proteins (*22*, *23*) activated the TLR4-mediated signaling pathway. Unfortunately, some of these TLR4-mediated activations were attributed to the contamination of LPS (*24*). Thus, we confirmed the involvement of LPS in the β-casein (1−28)-mediated activation with two methods. First, we assessed β-casein (1−28) solution with an LAL test kit. This assessment revealed no obvious gelatinization (data not shown). Lysate from horseshoe crab such as *Limulus polyphemus* is known to gelate in the presence of LPS (*25*). The negative gelatinization in the LAL test indicates that the β-casein (1−28) contains no more than 0.25 EU/mL LPS. Second, we confirmed whether or not the mitogenic activity of β-casein (1−28) was affected by PMB. As shown in **Figure 4**, no significant changes in the mitogenic effect of β-casein (1−28) were observed in the presence of PMB. PMB binds lipid A structure of LPS and is shown to disturb its mitogenic activity in mouse spleen cell cultures (*26*). Considering these results, it is suggested that the mitogenic effect of β-casein (1−28) is not due to LPS.

Figure 5A shows that β-casein (1−28) enhances IL-6 expression by CD19+ cells. TLR-4 signaling is known to enhance the expression of several cytokines such as IL-6, IL-10, and tumor necrosis factor (TNF)-α (*27*, *28*). In this respect, we found that β-casein (1−28) also enhanced IL-10 and TNF-α

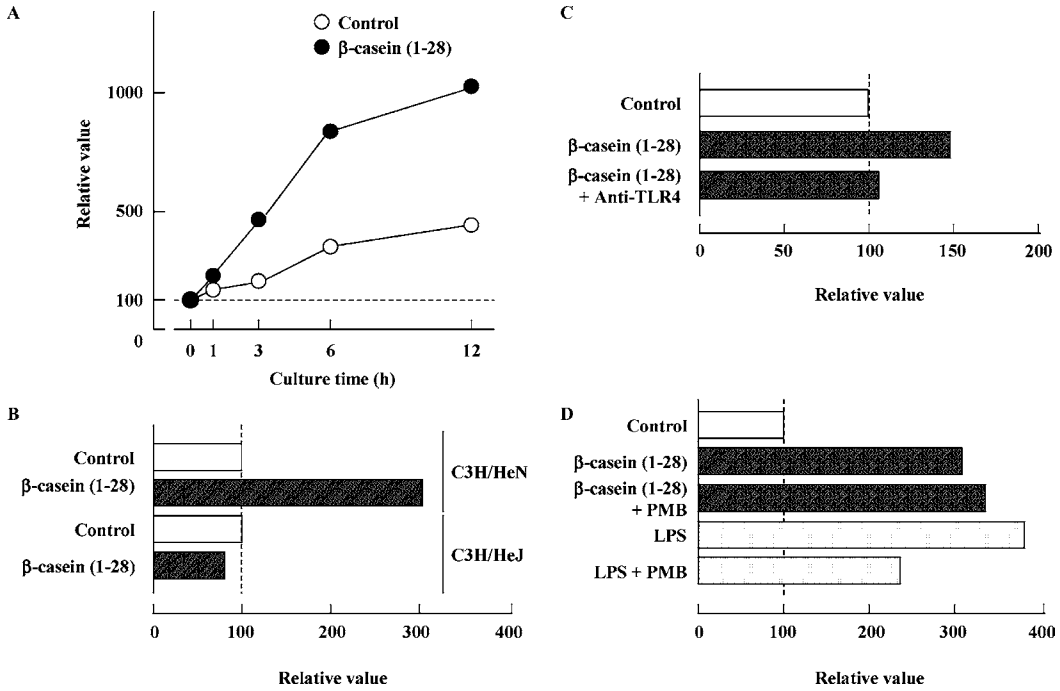

Figure 5. Effect of β-casein (1–28) on IL-6 expression of CD19$^+$ cells. All data were represented as typical values in relation to the value in β-casein (1–28)-free culture ($n = 3$ for **A**, **C**, and **D**, and $n = 2$ for **B**). (**A**) Time course expression of IL-6 in CD19$^+$ cells from C3H/HeN mice. The cells were stimulated with 500 μg/mL β-casein (1–28) for 72 h. (**B**) Effect of β-casein (1–28) on proliferative responses and cytokine expressions of CD19$^+$ cells from C3H/HeN or C3H/HeJ mouse. Cells were stimulated with 500 μg/mL β-casein (1–28) for 72 h. (**C**) Effect of β-casein (1–28) on IL-6 expression of CD19$^+$ cells from C3H/HeN mice in the presence of anti-TLR4 antibody. Cells were stimulated with 125 μg/mL β-casein (1–28) in the presence or absence of 4.2 μg/mL anti-TLR4 antibody for 72 h. (**D**) Effect of β-casein (1–28) on IL-6 expression of CD19$^+$ cells from C3H/HeN mice in the presence of PMB. Cells were stimulated with 500 μg/mL β-casein (1–28) or 0.5 μg/mL LPS in the presence or absence of 50 μg/mL PMB for 72 h.

expression by CD19$^+$ cells (data not shown). Thus, we examined whether IL-6 expression of C3H/HeJ mouse-derived CD19$^+$ cells were irresponsive to β-casein (1−28). **Figure 5B** indicates that the expression of IL-6 is notably diminished, as expected. Furthermore, competitive inhibition in β-casein (1−28)-induced IL-6 expression of CD19$^+$ cells from C3H/HeN mice was observed by treating β-casein (1−28) with anti-TLR4 antibody, while the expression was not affected by the treatment with PMB (**Figure 5C,D**). Therefore, it was also suggested that IL-6 expression of CD19$^+$ cells by β-casein (1−28) was mediated by TLR4.

As mentioned above, at least the tripeptide sequence SerP-X-SerP has been revealed as necessary for the mitogenic action of β-casein (1−28) (*14*). Kitazawa et al. reported that some phosphorylated saccharides secreted from bacteria are known to have mitogenic activity to mouse spleen B cells (*29*−*31*), which were the primary population of the CD19$^+$ cells (*32*). In addition, some of the saccharides require phosphate groups for their mitogenic action (*30*, *31*). Although the relationship between these phosphorylated mitogenic structures and TLR is still unclear, the phosphoserine residues of β-casein (1−28) may act similarly to the phosphate groups in these phosphorylated mitogens.

CPP are now sanctioned in Japan as a food ingredient for specified health uses due to their function of increasing the bioavailability of calcium in the human intestinal tract. Therefore, we previously showed that CPP-III enhanced IgA proliferation in humans as well as mice (*33*). The enhanced levels of IgA by CPP-III are explainable because IL-6 induces both proliferation and IgA production of B cells (*34*). However, LPS as a TLR4 ligand is a major pyrogen (*35*). We must explain why β-casein (1−28) can exert the immunoenhancing activity via TLR4 without pyrogenic side effects. A recent study demonstrated that the distinct structure of LPS triggers a different class of immune response (*36*). This fact indicates that TLR4-mediated signal outputs have a certain degree of diversity. Interaction of β-casein (1−28) with TLR4 may result in the promotion of proliferation and IL-6 production of CD19$^+$ cells for enhancing intestinal IgA at an appropriate level in humans.

In conclusion, we found that β-casein (1−28) enhanced proliferation and IL-6 production of CD19$^+$ cells and that both enhancements were mediated by TLR4. The mechanism revealed in this study is expected to encourage the understanding of β-casein (1−28)-containing food as useful immunoregulatory products for human health.

ABBREVIATIONS USED

CPP, casein phosphopeptide(s); SerP, phosphoserine; RP-HPLC, reverse-phase high-performance liquid chromatography; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; LAL, limulus amebocyte lysate; PMB, polymyxin B; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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