

Murine Antigen-Presenting Cells Are Multifunctional In Vitro Biosensors for Detecting the Immunoactive Potential of Bovine Milk Products¹

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ABSTRACT Antigen-presenting cells (APCs) are multifunctional components of the immune defense system. In this study, murine APCs were used as biosensors to detect immunologically active components of bovine milk and colostrum. By measuring changes in cell surface protein markers [major histocompatibility complex II, cluster designation (CD)40, CD86] and cytokines (tumor necrosis factor- α and interleukin-10) associated with APC activation, we identified a number of compounds that are immunoactive. The mouse macrophage cell line MH-S offered a simple and robust target for identification of immunoactives. The assay was shown to be adaptable for measuring immunoenhancing or immunosuppressive substances. Large-scale screening of milk extracts using this bioassay has the potential to identify substances that could be developed into nutraceuticals or pharmaceutical-grade immunotherapeutics. J. Nutr. 135: 2651–2656, 2005.

KEY WORDS: • antigen-presenting cell • milk • immunoactive • cytokines • CD markers

To achieve maximum reproductive success, mammals make a large physiological investment in their offspring. Colostrum and milk are complete nutritional substances that provide biologically active factors that enhance the survival of infants (1). These are particularly important for the poorly prepared immune system of the neonate (2) and help the naïve immune system while it develops in the environment into which the infant was born (3). Bovine colostrum is ideally suited for supporting the neonatal immune system of calves. Not only does it have high nutritional value, but it contains immunoglobulins (4) and other immune-enhancing factors (3,5). Many as yet undiscovered immune-regulating factors may be present in bovine colostrum and milk.

New immunomodulatory roles for milk proteins are steadily being discovered. For example, a bovine milk–derived protein with growth factor–like activity and homology to the cytokine transforming growth factor β (TGF β)³ was reported; this protein can suppress human lymphocyte proliferation (6). In mice, TGF β was studied in animals genetically deficient for this growth factor (7); neonatal mice developed chronic inflammation of the lower intestine, although this remained suppressed for longer in mice that received maternal milk containing immunosuppressive TGF β analogs.

In addition to whole bioactive proteins and lipids, milk and colostrum also contain peptides, which are activated only upon digestion and cleavage from the parent molecule. These are known as “encrypted peptides.” Many bioactive peptides with immunomodulatory effects are encrypted in bovine milk proteins (8).

The discovery of new, biologically active products in milk or colostrum has become a major area of research in the dairy industry. Platform technologies have been developed by many companies to produce libraries of potentially biologically active compounds. To identify the novel, commercially viable compounds within the large number of compounds produced in a library, robust screening procedures are required. Simple bioassays can be developed to screen the library for promising products, which can then be subjected to more specific, labor-intensive functional assays, in vivo studies, and clinical trials.

The present study aimed to develop such a bioassay, with the ability to detect immunomodulatory activity of protein extracts from bovine milk. The desired target compounds were those with the potential to have an effect on multifunctional cells of the immune system, and consequently, the immune response as a whole. To study whether a compound is a modulator of the immune system, the ideal place to start is with antigen-presenting cells (APCs). The 2 primary APCs of the immune system are dendritic cells (DC) and macrophages. APCs are the multifunctional directors of the immune response. They are highly responsive to their environment and relay messages instructing other arms of the immune system e.g., T cells and B cells, to respond in an appropriate way. These messages can be membrane-anchored cell surface molecules that facilitate direct interactions between APCs and T cells (costimulatory molecules), or soluble proteins (cytokines) that act like hormones and can signal systemically. Costimu-

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³ Abbreviations used: APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cells; CD, cluster designation; CMI, cell-mediated immunity; DC, dendritic cell; EU, endotoxin unit; FACS, fluorescence-activated cell scanning; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TGF β , transforming growth factor β ; TNF α , tumor necrosis factor- α .

latory molecules such as cluster designation (CD)40, CD80, and CD86 are produced by the APC to aid their interaction with the T cell upon antigen presentation (9). Low levels of these molecules may be present on the APC before antigen presentation. After antigen processing, gene expression is up-regulated and the number of molecules present on the cell surface increases markedly. In addition to upregulating the expression of cell surface molecules, APCs can secrete soluble protein cytokines. Interleukin (IL)-10 and tumor necrosis factor- α (TNF α) are examples of anti-inflammatory and proinflammatory cytokines, respectively, expressed by APCs after activation. The cell surface markers and cytokines act together to carefully control all parts of the immune system as well as the type of response generated.

IL-10 production by APCs inhibits the generation of cell-mediated immune responses and is considered anti-inflammatory. It is associated with humoral or Type 2 immune responses. TNF α is a strong inducer of inflammatory responses and is associated with cell-mediated immunity (CMI, or Type 1) responses. Immunopathology can arise when the balance between Type 1 and Type 2 responses is not tightly controlled, for example, in chronic inflammation (10).

Several immortalized macrophage cell lines are available that proliferate rapidly and remain stable over many passages. These cells are relatively easy to use and could potentially provide an experimentally accessible "read out" via the cell surface markers and cytokine secretion. Here we describe the use of a macrophage cell line (MH-S), in addition to murine bone marrow-derived DCs (BMDCs), to screen compounds isolated from bovine milk/colostrum for immune-regulatory activity.

MATERIALS AND METHODS

Milk products. The bovine milk extracts investigated in this study were a carefully selected group of well-characterized, discrete milk protein fractions and protein hydrolysates that covered the entire proteome of milk and colostrum. They were assayed as either spray-dried or freeze-dried powders. The exact composition cannot be revealed for commercial reasons. Products were assayed for lipopolysaccharide (LPS) contamination and the concentration of endotoxin was $<0.15 \mu\text{g/kg}$ [$=1.25$ endotoxin unit (EU)/g, where $10 \text{ EU} = 1 \text{ ng}$]. The main test products were coded 1 and 2: Product 1 was a heat-treated skim milk powder and Product 2 was milk protein concentrate with 80% protein, 15% IgG, and no lactose. In addition, a further series of products coded A–L were investigated in a limited number of assays: Product A was an ion exchange whey protein fraction; Product B was a casein glycomacropeptide; Products C, D, E, and F were different ion exchange whey protein fractions; Products G and H were whey protein hydrolysates; Product I was a 70% whey protein concentrate; Product J was a colostrum milk protein concentrate; Product K was a novel milk product sialyl oligolact; and Product L was a colostrum whey protein concentrate.

Culture of the macrophage cell line MH-S. The cell line MH-S (ATCC number CRL-2019) was obtained from ATCC. A total of 5×10^6 MH-S cells were seeded in a 75-cm² falcon flask (BD Falcon) with 20 mL warm DMEM + 5% fetal calf serum (FCS) + L-glutamine (200 mmol/L) (all from Life Technologies/Gibco) and incubated at 37°C with 10% CO₂. Cells were routinely maintained at $<80\%$ confluence. To harvest cells for use in bioassays supernatants were removed from the adherent cells before the addition of 2 mL PBS + 2 mmol/L EDTA, and the culture was left at 37°C for 10 min. Vigorous pipetting helped dislodge cells, which were then washed in fresh serum-containing medium before counting.

Preparation of murine BMDCs. BALB/c mice (6–8 wk old) were obtained from the University of Otago Department of Animal Laboratory Sciences. These mice were used to produce a source of bone marrow cells, under approval from the University of Otago Animal Ethics Committee. Mice were killed humanely and bone

marrow was flushed from dissected femurs and tibias with 5% FCS in PBS. The RBCs were lysed with ammonium chloride and the remaining cells washed 3 times in PBS. Cells were plated at 2×10^6 cells/mL in 6-well plates and cultured in DMEM containing 20 $\mu\text{g/L}$ of granulocyte macrophage colony-stimulating factor (GM-CSF; derived from an *Escherichia coli* expression system). The cultures were fed on d 4 and 6 by replacing $\sim 75\%$ of the medium. On d 6–7, nonadherent cells were harvested as BMDCs.

Culture of APCs with bovine milk extracts. Harvested cells were counted and resuspended in culture media at 9×10^5 cells/mL. Milk protein extracts in powder form were diluted to a concentration of 2 g/L in appropriate cell culture media immediately before use. Serial dilutions were made to give appropriate concentrations. Cell suspensions were added 1:1 to media containing diluted test extract or control, and cultured in a 12-well tissue culture plate (Nunc). Control treatments included cells in medium only (negative) and a positive control of interferon γ (IFN- γ) at a concentration of 5 $\mu\text{g/L}$ plus LPS from *E. coli* 026:B6 (Sigma) at a concentration of 1 mg/L. Cells were incubated at 37°C with 10% CO₂ for 24 h before being harvested for cell surface marker staining.

Detection of cell surface markers by fluorescence activated cell scanning (FACS). Cell suspensions were transferred to tubes for FACS analysis. Cell suspensions were pelleted and resuspended in 1 mL ice-cold FACS Buffer (Dulbecco's PBS containing 5% FCS and 0.01% sodium azide). Aliquots (100 μL) of diluted fluorescence-labeled anti-murine antibodies [anti-CD40, anti-CD86, anti-major histocompatibility complex (MHC) II] or appropriate isotype controls (cat #553930 and 553989) were added to separate tubes (all antibodies PharMingen). Cells were incubated on ice for 30 min. Cells were washed in FACS buffer and then resuspended in 200–400 μL FACS Buffer. The presence of labeled cell surface markers was detected using a FACScalibur flow cytometer (Becton Dickinson). Results were analyzed using CellQuest or CellQuestPro software (Becton Dickinson).

Preparation of supernatants from pulsed cells. Cells were suspended in media at 9×10^5 cells/mL. Media containing milk extracts (2 g/L) or control treatments were then added to the cells in a 1:1 ratio in a 12-well plate (Nunc). Control treatments included cells in medium only (negative) and a positive control of IFN- γ (5 $\mu\text{g/L}$) plus LPS (1 mg/L). Cells were incubated at 37°C with 10% CO₂ for 6 h after which media was removed from the wells, leaving the adherent cell layer behind. Wells were then rinsed with medium, and 2 mL of fresh medium was replaced into each well. Samples were then incubated for a further 24 h at 37°C with 10% CO₂ after which the supernatants were harvested and prepared for cytokine analysis using an ELISA. Cytokines assayed for were IL-10 and TNF α .

Cytokine detection by ELISA. Rat anti-mouse cytokine capture antibody pairs (rat anti-mouse IL-10 #551215, rat anti-mouse TNF #551225; PharMingen) were diluted to 1–4 mg/L (1/5000) in coating buffer. ELISA plates (BD Falcon) were coated with 50 μL /well of diluted antibody and incubated overnight at 4°C. Plates were then washed 4 times in wash buffer to remove unbound antibody. Non-specific protein binding was prevented by adding 200 μL blocking buffer (PBS containing 10% FCS) per well and incubating for a further 2 h at room temperature. Blocking buffer was removed by washing 4 times in wash buffer. Standard cytokines [IL-10 and TNF α (PharMingen)] and samples were diluted in blocking buffer and 100 μL added per well before plates were covered and incubated overnight at 4°C. Plates were then washed 4 times in wash buffer before the addition of 100 μL /well of biotinylated detection antibodies (rat anti-mouse IL-10 #554423, rat anti-mouse TNF α cat #554415; PharMingen), which were diluted 1:500 in block buffer. The plates were incubated at room temperature for 45 min, then washed 4 times in wash buffer. Streptavidin horseradish peroxidase (Zymax Grade™, ZYMED Labs) was diluted 1:3000 in blocking buffer and 100 μL was added per well. After 30 min of incubation at room temperature, plates were washed 4 times in wash buffer. Trimethylbenzidine substrate (TMB kit, ZYMED) was added at 100 μL /well and left for color to develop; the reaction was stopped by the addition of 100 μL of 1 mol/L H₂SO₄ to each well, and the optical densities were read at 540 nm using a Microplate Reader (Model 550, BioRad).

Assays for suppressive activity of milk compounds on stimulated APCs. To determine whether any of the milk extracts could have a suppressive effect on stimulated APCs, 9×10^5 cells/mL were first stimulated by incubation in media containing IFN- γ (5 μ g/L) at 37°C with 10% CO₂ for 6 h. Following this, medium only or media containing milk extracts (2 g/L) were added 1:1 per well and incubated at 37°C with 10% CO₂ for 24 h. Cells were harvested and prepared for FACS analysis as described above.

Data interpretation and statistical analysis. For the interpretation of qualitative data (such as cell staining after surface fluorescence labeling), a sample was considered positive if the expression of molecules induced by a treatment was at least 2 SD above the standard negative control (skim milk). For the statistical evaluation of numerical data, such as the quantitative effect of different treatments on cell responses, one-way ANOVA was used (SPSS). Dunnett's post hoc test was employed to identify significant differences between controls (no treatment) and the various treatments. In all cases, $P < 0.05$ was taken to identify significant differences.

RESULTS

Expression of activation markers on MH-S cells in response to IFN- γ and milk compounds. Changes in expression of the APC costimulatory molecule CD40 were determined in MH-S cells in the absence (Fig. 1A) and presence (Fig. 1B) of stimulation with IFN- γ , and compared against the effects of a milk compound previously found to have immunostimulatory activity (Fig. 1C). The milk compound activated APCs, causing upregulation of CD40 expression on cells in a manner similar to that of immune stimulant IFN- γ , as shown by a shift to the right in the fluorescence intensity histogram.

Analysis of the percentage of total cells with upregulated activation markers (CD40, CD86, or MHC II) in response to positive control stimulation (IFN- γ or LPS) or to 2 milk extracts (1 with immunomodulatory activity [Product 2] and 1 without [Product 1]) indicated that these response patterns were broadly similar in MH-S cells (Fig. 2, upper panel) and mouse BMDCs (Fig. 2, lower panel). Results are representative of all positive samples tested; no sample was found to give positive results with one cell type and not the other. Although MH-S cells showed negligible expression of activation markers in response to the negative control, they strongly upregulated expression of CD40 ($P < 0.001$), CD86 ($P < 0.001$), and MHC II ($P < 0.001$) on the cell surface when treated with milk Product 2 or the positive control (IFN- γ) (Fig. 2, upper panel). There was no significant upregulation of expression of any marker after treatment with Product 1. Similarly, BMDCs were hyporesponsive to Product 1 and the negative control (Fig. 2, lower panel), with a positive staining frequency of $< 5\%$ of total cells for CD40. As with the MH-S cells, the known immunomodulatory milk extract (Product 2) caused significant increases in CD40, CD86 and MHC II expression on BMDCs ($P < 0.001$) (Fig. 2, lower panel).

The immunomodulatory activity detected in Product 2 was able to be titrated between 1 g/L and 40 mg/L (Fig. 3) at which point CD40 levels were still significantly higher than those of the negative control ($P < 0.001$). Product 2 was also immunomodulatory for CD86 and MHC II, but over a smaller range (1 g/L to 200 mg/L; Fig. 3). There was no difference in activation marker upregulation after treatment with 8 mg/L.

To test its generality, the MH-S assay was used to screen 12 additional milk extracts (A–L). CD40 expression increased after treatment of MH-S cells with extracts A ($P < 0.001$), E ($P < 0.001$), and I ($P = 0.001$) (Fig. 4). The same extracts (A, E, I + K) also induced significantly increased CD86 expression ($P \leq 0.001$). No upregulation of CD40 or CD86 occurred after treatment with any of the other milk extracts. Extracts E and

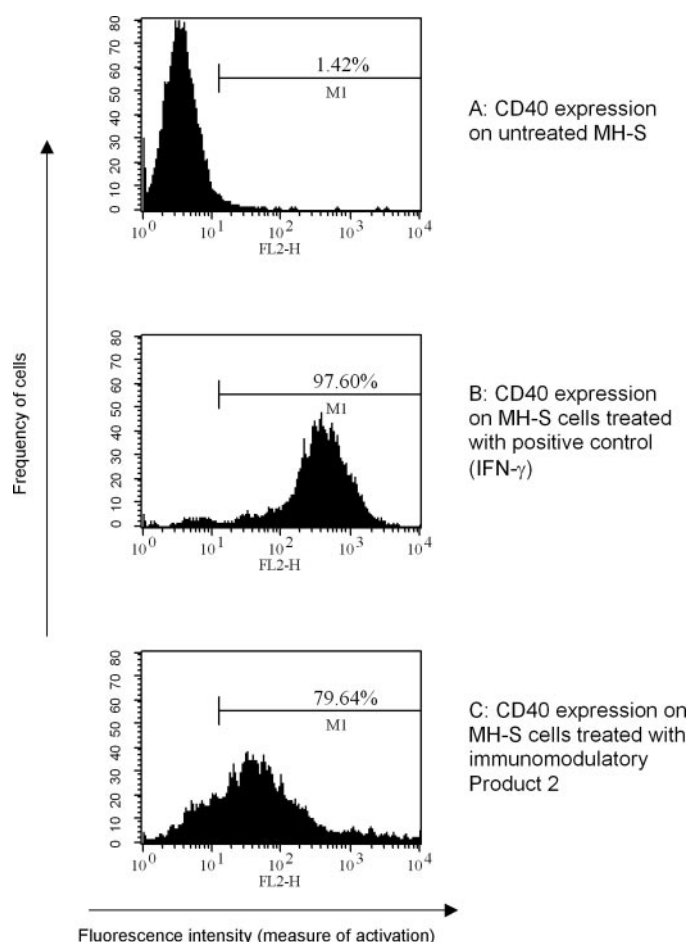


FIGURE 1 Milk products can activate murine APCs in vitro. Fluorescence intensity histograms, depicting MH-S cells staining positively for CD40 in the absence of pretreatment (A), after treatment with IFN- γ (B), and after treatment with an immunomodulatory milk compound (Product 2) (C). Note the prominent shift to the right in response to IFN- γ or Product 2, indicating immune activation (percentage figures represent the % of cells positive beyond the cutoff FL2 value of 10^1 fluorescence units).

I also induced the upregulation of MHC II ($P < 0.05$). MHC II was not upregulated after treatment with any of the other milk extracts.

Cytokine expression by APCs. Interleukin-10 production by MH-S cells was measured after treatment with the 12 milk extracts. A significant level of IL-10 was detected in the supernatants of cells treated with samples A ($P < 0.001$) and F ($P < 0.001$) compared with the negative control (Fig. 5). No other compounds induced significant levels of IL-10. No TNF α was detected in supernatants of cells treated with any of the milk extracts. The MH-S cells treated with IFN- γ + LPS did, however, produce a large amount of TNF α (5.4 ± 0.4 μ g/L), indicating that these cells were capable of producing this cytokine.

Ability of milk extracts to modulate cell surface markers on stimulated APCs. MH-S cells that had been preactivated by exposure to 50 ng/L IFN- γ for 6 h before coculture for a further period of 24 h with milk extracts, demonstrated a degree of regulation of cell surface molecule expression (Fig. 6). After stimulation with IFN- γ , 6 of the milk extracts (A, D, E, G, I, and K) induced some degree of synergistic upregulation of CD40 (Fig. 6, upper panel). CD86 expression on preacti-

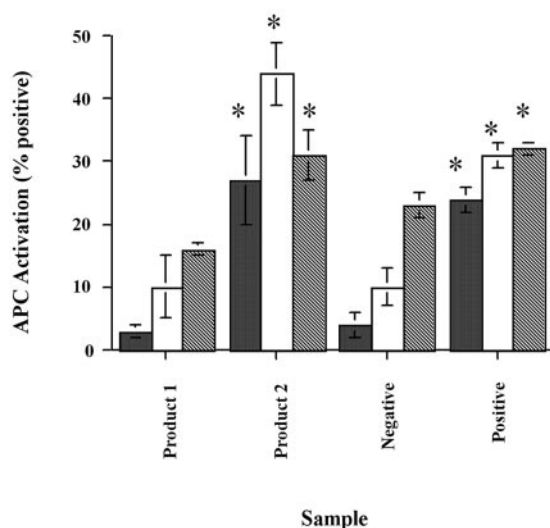
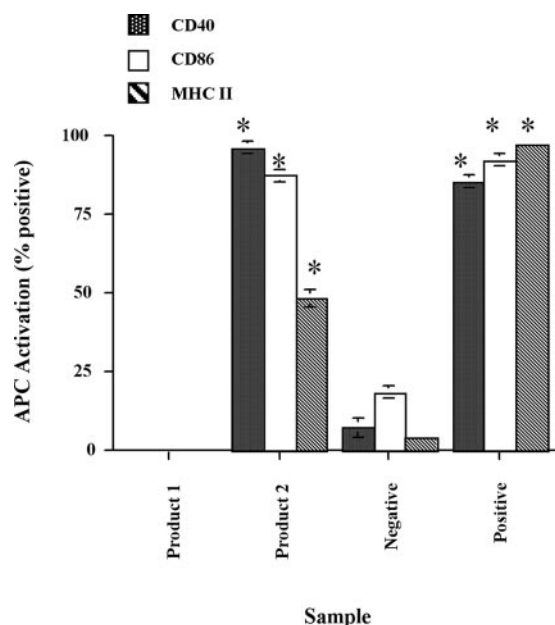


FIGURE 2 Cell surface marker expression in the cell line MH-S (upper panel) and in murine primary bone marrow-derived dendritic cells (lower panel) is modulated by milk products in vitro. A comparison was made of responses by a mouse macrophage cell line MH-S (upper panel) and mouse bone marrow derived dendritic cells (lower panel) to 2 milk compounds. Responses were measured by the upregulation of the surface markers CD40 (shaded bars), CD86 (open bars) and MHC II (hatched bars). Product 1 showed no immunoactivity, whereas Product 2 showed immunoactivity equaling the positive control of LPS and IFN- γ . A negative control of culture medium alone is shown. Values are means $\pm$ SD, $n = 3$ individual replicate cell cultures. *Different from the negative controls, $P < 0.001$.

vated cells was also strongly induced by extracts A, E, I, and K. None of the extracts upregulated MHC II above the level induced by pretreatment with IFN γ . Only 1 compound (C) showed significant suppressive activity of MHC II expression on preactivated APCs (Fig. 6, lower panel).

DISCUSSION

Certain milk proteins and hydrolysates were identified previously as having the ability to influence the immune response

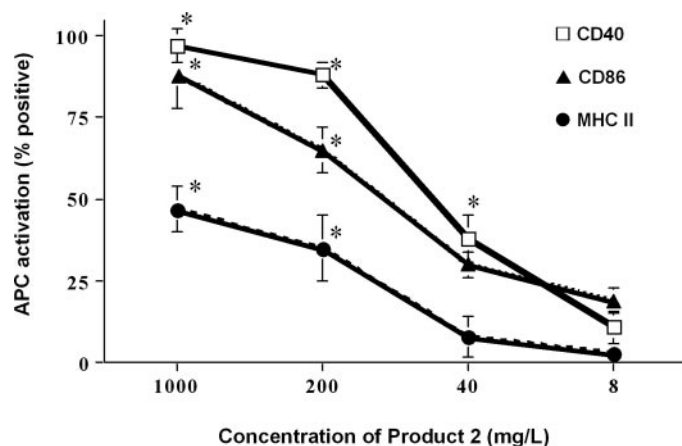


FIGURE 3 Titration of the immunomodulatory effects of bovine milk Product 2 in murine APCs (MH-S cells). The known immunomodulatory milk protein Product 2 was tested in 5-fold serial dilution for immunoactivity against MH-S cell in vitro. Values are means $\pm$ SD, $n = 3$ individual replicate cell cultures. *Different from the negative controls, $P < 0.001$.

(3,4,8,11–14). In this study, we used APCs as representative multifunctional cells of the immune system, suitable for use as biosensors for detecting immunomodulatory activity. We screened a library of milk extracts to isolate compounds with immunomodulatory activity using simple and robust bioassays. As a general approach, initial screening for immunoactive compounds can be carried out on complex compounds to identify those fractions worth examination; these fractions can then be progressively purified and their bioactivity tracked. We selected 12 rather crude preparations in this study to show proof of concept because immunoactive compounds may be discrete chemical compounds or may instead result from a

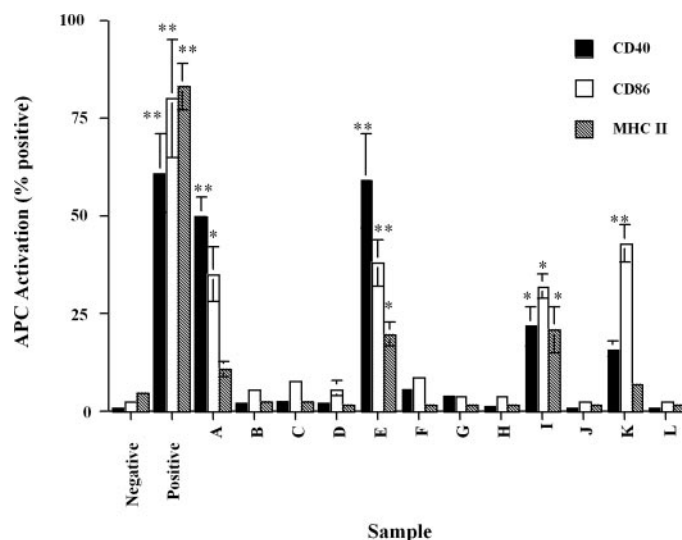


FIGURE 4 Measuring cell surface activation marker expression on murine APCs for the simultaneous screening of multiple milk components for immunomodulatory activity. The immunoactivity of 12 milk extracts (A–L) was assessed by measuring the upregulation of the indicator molecules CD40, CD86, and MHC II. Samples were compared with a negative control of cells cultured in medium alone and a positive control of cells cultured with LPS and IFN- γ . Asterisks indicate difference from the negative controls: * $P < 0.005$; ** $P < 0.001$.

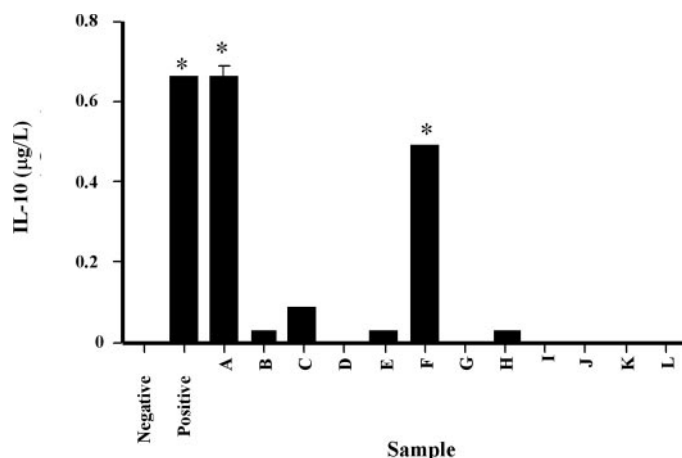


FIGURE 5 Measuring cytokine production by murine APCs for the simultaneous screening of multiple milk components for immunomodulatory activity. The production of IL-10 by the murine macrophage cell line MH-S was measured by ELISA. IL-10 expression in response to milk extracts A–L was compared with a negative control of cells cultured in medium alone and a positive control of cells cultured with LPS and IFN- γ . *Different from the negative controls, $P < 0.001$.

synergy of several milk components (13,14). Large numbers of fractions require assaying before the decision can be made concerning which compounds might feasibly be developed further to produce pharmaceutical or nutraceutical products.

APCs are ideal cells to use as biosensors because they respond to their surroundings in an easily measured manner. APCs recognize and take up antigens from their environment, and present these to T cells to direct the appropriate immune response. To achieve successful T-cell activation, the APC is required to deliver 2 signals to the T cell. The first results from engagement of the T-cell receptor by antigen fragments presented on molecules of the MHC II. To facilitate this process, activated APCs greatly increase the number of MHC II molecules on their surface in response to certain environmental signals. The second signal is delivered by a range of costimulatory molecules. These can be either surface bound as with CD40 and CD86 or secreted, as is the case with cytokines such as TNF α , IFN- γ , and IL-10. By monitoring the expression of these “activation markers” by APCs, we were able to identify a number of milk extracts with the ability to activate these cells.

A macrophage-derived murine cell line, MH-S, was compared with primary DCs, cultured from mouse bone marrow, to determine whether they responded in a similar manner. MH-S cells are a self-renewing, immortalized cell line that is simple to grow in culture. DCs are considered to be highly sensitive, professional antigen-presenting cells (15), but they are difficult to isolate and require careful handling to prevent them becoming activated before treatment with bioactives. Thus, they can produce variable results, making them less appropriate for a large-scale screening process in a biotechnology setting than a cell line such as MH-S. Our data suggest that, under the conditions tested for in these experiments, the MH-S cell line can be substituted for BMDCs without loss of assay sensitivity.

The response of APCs to particular milk extracts might give an indication of the type of condition/disease that may be treatable with particular extracts. For example, extracts with immunosuppressive activity could potentially be developed into a nutraceutical or pharmaceutical to aid in suppression of inflammatory autoimmune diseases such as rheumatoid arthritis or asthma. Extracts that stimulate CMI could be developed

as treatments for infections, some types of cancer, and conditions in which people suffer from immunosuppression.

Four extracts in this study had immunoactivity. Although cytokines such as IFN- γ are active in nanogram to picogram amounts, milk extracts are required in the microgram to milligram range. This may be due to the activity residing in a minor subfraction of these complex extracts; on the other hand, the immunoactive molecules could be relatively non-specific or target low-affinity receptors. Further subfractionation of positive compounds to obtain pure fractions will elucidate which option is correct. The relative expression of surface activation markers differed between samples, with CD40 appearing to give the clearest and most consistent differences between foreground and background.

Although none of the extracts induced TNF α production by MH-S cells, 2 induced IL-10, which downregulates activation markers on APCs (16). It is interesting to note that sample F induced IL-10 production in the MH-S cells but little upregulation of CD40, CD86, or MHC II, whereas sample A induced IL-10 production but also strongly upregulated other activation markers. Sample C had a potent suppressive activity on MHC II expression but appeared to induce little IL-10

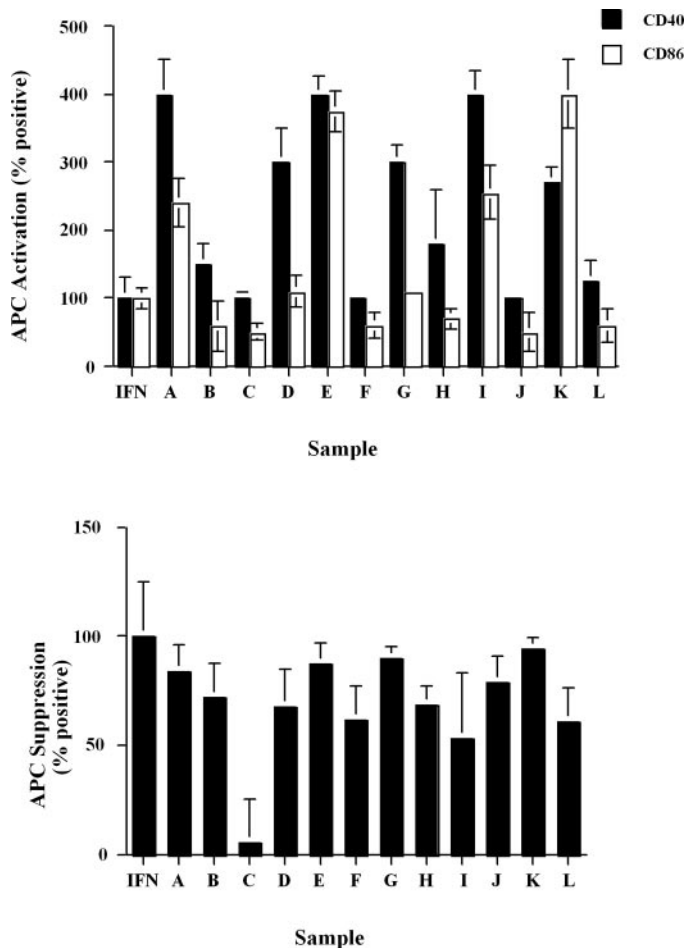


FIGURE 6 Use of biosensor assays to detect the immunomodulatory potential of milk components on IFN- γ preactivated murine APCs. *Upper panel:* upregulation of activation markers CD40 and CD86 after treatment of IFN- γ prestimulated cells with milk extracts. Values are expressed as a percentage of the value obtained using IFN- γ (5 μ g/L) to prestimulated cells. *Lower panel:* the suppression of MHC II expression on APCs after treatment of prestimulated cells with milk extracts. Values are representative of 2 experiments.

expression. This suggests that these complex compounds contain numerous and possibly antagonistic activities that will become obvious only with further purification (3).

We also adapted the assay to use activated APCs as the starting point and then looked for substances that would suppress or further enhance (act synergistically on) the expression of activation markers. Interferon- γ is an inducer of APC activation markers; thus, it was used to preactivate target cells. One milk-based product (C) had strong suppressive activity on IFN- γ preactivated cells, whereas extracts A, E, I, and K appeared to act synergistically with IFN- γ to upregulate the expression of activation markers. It is not surprising that these same 4 extracts were identified as being active on unstimulated APCs as well. Two samples (D and G) were able to upregulate CD40 but not CD86. The importance of differential expression of these markers will require further investigation.

Although there are many other activation markers that could be used to measure APC activation, this would invariably increase the complexity of the assay without providing a corresponding increase in information. For example, CD80 expression tended to mimic CD86 and CD40 expression in this study (data not shown). Because time and expense can be saved by minimizing the number of markers measured, assaying for 1 or 2 representative markers is likely to provide sufficient information to identify most compounds with immunoactivity on APCs.

Although food-grade cow's milk is pasteurized before the separation of protein extracts, the possibility of bacterial products such as LPS being present in the samples must be considered. LPS is an endotoxin and is a potent activator of APCs. Indeed, a recent study indicated that bacterial LPS contamination can be a major contributor to the perceived immunoactivity of bovine milk proteins (17). However, several lines of evidence suggest that microbial products such as LPS were not an important factor in our experiments. First, despite a common separation technique only a few (4 of 12) of the products showed any ability to activate target APCs. If microbial contamination was a major issue, we would have expected the majority of samples to have been positive in our assay system. LPS is a potent inducer of TNF α production by APCs. In our experiments, no TNF α production by MH-S cells was detected in response to coculture with milk proteins alone, whereas the LPS positive control induced large amounts. We found that the induction of cell surface, costimulatory molecules, in the MH-S cell line, is insensitive to LPS unless IFN- γ is also present. This argues against LPS being responsible for the activation of MHC II, CD40, or CD86. Finally, samples were tested independently at ESR (the National Testing Laboratory in New Zealand) for endotoxin contamination, and the concentration of endotoxin was <0.15 ng/g. When we tested these concentrations of LPS experimentally, as well as at

10-fold higher concentrations, on both MH-S and bone marrow dendritic cells, no stimulation of costimulatory molecules occurred.

The results of this study suggest that the expression of activation markers on APCs can be used reliably for testing milk extracts for activity, which could potentially have downstream effects on the immune response by virtue of the multifunctional action of APCs in vivo. Expression of these markers can be used to identify milk extracts with stimulatory or suppressive activities. Application of this process will facilitate the discovery of novel molecules in milk or colostrum before in vivo studies in animals and humans. Large-scale screening of milk extracts using the bioassay developed in this study has the potential to identify substances that could be developed into nutraceuticals or pharmaceutical-grade immunotherapeutics.

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