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Modulation of immune function by milk fat globule membrane isolates

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ABSTRACT

The nutritional value and characterization of minor milk components on mammalian immune function are not fully understood. The aim of this research was to test the ability of a milk fat globule membrane (MFGM) isolate to modulate murine immune function in vitro, by studying its effects on splenocyte proliferation, apoptosis, and cytokine production. Proliferation of spleen cells was not affected by the MFGM isolate; however, in the presence of polyclonal activators, the MFGM isolate suppressed cell proliferation. Results obtained by flow cytometry did not support programmed cell death as the cause of the MFGM immune-modulating capacity. A mode of suppression on the splenocyte activation process was suggested from a marked decrease in the production of IFN-γ and tumor necrosis factor-α cytokines, typical indicators of immune cell activation. The effect of MFGM on IL-4 secretion was significantly less than that for the other 2 cytokines. The activity exerted by the MFGM over concanavalin A-stimulated cells differed from that observed in cells treated with lipopolysaccharide, suggesting a different mode of action depending on the activator used. These results indicate the potential of MFGM extracts as functional ingredients with bioactive modulating capacity.
Key words: immunomodulation, milk fat globule membrane, antiinflammatory, bioactivity

INTRODUCTION

Milk represents a rich source of biologically active molecules that are able to influence a range of physiological functions. An increasing number of its components have been shown to provide immunological protection or other biological activities to neonates and adults. For example, several milk proteins and their enzymatically derived peptides have immunomodulatory properties (Debbabi et al., 1998; Cross and Gill, 2000; Eriksen et al., 2008) and have shown effects on lymphocyte proliferation and activation, cytokine secretion, antibody production, granulocyte and natural killer cell activity, and phagocytic activity (Cross and Gill, 2000; Gauthier et al., 2006).

Immunoregulation consists of both enhancement and suppression of the immune system, and the final immune response is a result of the balance between enhancing (helper) and suppressive influences (Krakauer and Clough, 1980). In this context, cytokines play an important role in mucosal antibody- and cell-mediated immunity. Following activation, naive T helper (**Th**) cells are divided into 2 main categories: Th_1 cells, which play a critical role in the regulation of cellular immunity through the secretion of IL-2, IFN-γ, and tumor necrosis factor (**TNF**)-α cytokines; and Th_2 cells, which regulate antibody-mediated immune response through the production of IL-4, IL-5, and IL-10 (Krakauer and Clough, 1980; Mao et al., 2007). Many factors influence this cell differentiation, including the dose of antigens, the nature and degree of co-stimulation, and the cytokine milieu surrounding the differentiating cells.

Nutrients might modulate immune maturation and responses and provide factors that influence the intestinal microbiome (Rutherfurd-Markwick and Gill, 2005; Zhou and Gao, 2010). Nutraceuticals enriched with milk-derived fractions are thus a target for commercialization, and hence, attention is being given to the characterization of dairy components with bioactive capacity (Cross and Gill, 1999; Benyacoub et al., 2011).

Biofunctionality studies on milk components (i.e., serum proteins and their respective hydrolysates) have either used commercially obtained fractions or highly purified individual components. In most cases, the primary sources have been not been well characterized or the possibility of contamination with other components has not been considered. When performing immunomodulatory studies, it is important to determine the likelihood of bacterial LPS presence on the isolates under study. Contamination might contribute to inconsistent literature data. For example, previous researchers reported that an LPS-free β-LG isolate from raw milk had no immune-stimulatory effect on murine bone

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marrow dendritic cells (Brix et al., 2003), contradicting previous findings.

In addition to whey proteins, components of the milk fat globule membrane (**MFGM**) display several biological activities such as antiviral, anticarcinogenic, and antimicrobial properties (Singh, 2006; Clare et al., 2008; Rasmussen, 2009), as well as important synergies with probiotics (Brisson et al., 2010; Benyacoub et al., 2011).

The native MFGM is a highly structured trilayer membrane that surrounds the native fat globules. Although it represents only 2 to 6% of the total mass of fat globules, it comprises between 60 and 70% of the total polar lipids in milk (Spitsberg, 2005; Singh, 2006). A recent patent highlights the immune-protective role of the MFGM extracts in infant formula (Benyacoub et al., 2011), but few studies have focused on the MFGM modulation of lymphocyte biology, and the role of MFGM in the development of the specific host-defense system remains to be clarified.

Considering current trends in product development that look for nutritional value along with the delivery of health benefits, it is desirable to define whether minor milk components and milk by-products have the potential to affect physiological functions, so that their incorporation into manufactured products will provide benefits to human health. For example, patients with chronic inflammatory diseases of the gastrointestinal tract may benefit from dietary products that can counteract the autoimmune host cellular responses and hence limit localized inflammation (Cross and Gill, 1999).

The aim of the present study was to better understand the mechanisms involved in the immune-modulatory capacity of an MFGM isolate from milk.

MATERIALS AND METHODS

Preparation of MFGM Isolate

Fresh raw milk samples were collected at the Elora Research Station (Guelph, ON, Canada) under sterile conditions, and isolation was carried out as previously described (Zanabria et al., 2013). For cell stimulation, the freeze-dried material was reconstituted using endotoxin-free water (2:1 wt/vol) and protein content was determined by the Lowry assay (DC Protein Assay, BioRad Laboratories, Hercules, CA) using BSA as a standard (BioRad). The amount of LPS was determined before all bioactivity analyses, using the Limulus Amebocyte Lysate Pyrochrome kit (Associates of Cape Cod Inc., Cape Cod, MA). Positive controls and endotoxin-spiked samples were included in the experiments.

Isolation and Culture of Spleen Cells from Healthy Animals

Female BALB/c mice, 6 to 8 wk old, were obtained from Charles River Laboratories (Montreal, QC, Canada). All mice were housed at the Central Animal Facility of the University of Guelph and studies were carried out in accordance with the Ethics Board (Animal Utilization Protocol No. 10R002, University of Guelph).

Cells were grown in RPMI-1640 medium (Gibco Life Technologies Corp., Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 1% (vol/vol) penicillin-streptomycin (dual antibiotic solution, 10,000 U/mL; Invitrogen Canada Inc., Burlington, ON), 2 m*M* L-glutamine, and 50 μ*M* 2-mercaptoethanol (Sigma Aldrich, Oakville, ON, Canada).

Mice were euthanized via CO_2 inhalation; the spleens were removed and single-cell suspensions prepared individually under aseptic conditions. Fat tissue was separated and spleens trimmed with a scalpel in 100-mm cell culture dishes (BD Falcon, Fisher Scientific Canada, Toronto, ON, Canada) containing 5 mL of complete medium. Further disaggregation was achieved by pressing the tissue with a syringe plunger and passing the cell suspension through a 70-μm cell strainer, after which it was centrifuged at 300 × *g* for 7 min at 20°C. Subsequently, erythrocytes were removed using the red blood cell lysing buffer Hybri-Max (Sigma Aldrich) at room temperature (20°C). The resulting leukocyte suspension was washed twice with RPMI medium (300 × *g*, 7 min, 4°C) and the cell number was adjusted to the required cell density. Viability was assessed by trypan blue exclusion and was always >90%.

In Vitro Analysis of Splenocyte Proliferation

Cell proliferation was used as a marker of activation. The capacity of native MFGM isolates to affect cellular immune function was determined by its inclusion in in vitro culture of murine splenic leucocytes. Two different concentrations of the isolate were tested (50 and 100 μg/mL of MFGM based on protein), based on previous studies showing substantial levels of bioactivity at these 2 concentrations (Zanabria et al., 2013).

A suspension containing freshly isolated splenocytes (100 μL, 5×10^4 cells) was seeded (in quadruplicate) in a 96-well tissue culture plates (Corning Inc., Corning, NY). Medium (50 μL) containing either MFGM or one of the mitogenic compounds LPS and concanavalin A (**Con A**) was added to the wells. The addition of Con A (Sigma) at 2.5 μg/mL or LPS from *Escherichia coli* 0111:B4 (Sigma) at 20 μg/mL would induce cell proliferation. Prior experiments using LPS (0–50 μg/mL)