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Original article

The production and characterization of anti-bovine CD14 monoclonal antibodies

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Abstract – To characterize further the chemical and biological properties of bovine soluble (bos) CD14, a panel of ten murine monoclonal antibodies (mAb) reactive with recombinant (r) bosCD14 were produced. A sandwich ELISA, using murine mAb and rabbit polyclonal antibodies reactive with rbosCD14 was developed. All the mAb were reactive by ELISA with baculovirus-derived rbosCD14 and they recognized rbosCD14 (40 kDa) by western blot analysis. The mAb also identified by western blot sCD14 (53 and 58 kDa) in milk and blood and sCD14 (47 kDa) in a lysate of macrophages obtained from involuted bovine mammary gland secretions. Analysis by ELISA of whey samples after intramammary injection of lipopolysaccharide (LPS) (10 μg) revealed increased sCD14 levels between 8 to 48 h after injection. Flow cytometric analysis showed that the mAb bound to macrophages isolated from involuted mammary gland secretions and mouse macrophages but not to swine or horse monocytes. Addition of anti-rbosCD14 mAb to monocytes stimulated with LPS reduced in vitro production of TNF-α. The anti-rbosCD14 antibodies generated in this study will be useful in studying CD14, an accessory molecule that contributes to host innate recognition of bacterial cell wall components in mammary secretions produced during mastitis.

CD14 / monoclonal antibodies / ELISA / LPS / mastitis

1. INTRODUCTION

CD14 was first described as a monocyte/macrophage differentiation antigen that is attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor [17]. Membrane CD14 (mCD14) is a 55 kDa glycoprotein which binds lipopolysaccharide (LPS) and initiates cell activation. This receptor is abundant on the cell membrane of monocytes and macrophages and is present at a lower density on polymorphonuclear neutrophil leukocytes (PMN) [1]. Soluble CD14 (sCD14) has been detected in bovine milk [23], and in human serum and urine [4, 17, 26]. Membrane CD14 is an accessory receptor for LPS that activates monocytes, and is capable of binding low concentrations of LPS that are complexed to serum-derived LPS-binding protein (LBP) [39].

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Soluble CD14 exists in at least two isoforms which range in size from 48–56 kDa [8]. Soluble CD14 found in human serum has been attributed to the shedding of mCD14 from monocytes, macrophages and PMN [8, 20] Studies with human monocytes and monocytic cell lines have shown that mCD14 is released via protease-dependent or independent pathways. Some isoforms originate from shedding of mCD14, while others are secreted after synthesis by monocytes and macrophages [5]. Soluble CD14 present in the serum/plasma and urine of nephritic patients mediates activation of cells not bearing mCD14 including epithelial cells and endothelial cells [2, 15]. Macrophages are the predominant cell type in milk from uninfected bovine mammary glands whereas PMN predominate in mastitic milk. Bovine macrophages and PMN in milk express mCD14 on their cell surface [28] and are potential sources of sCD14.

Recognition of LPS involves soluble proteins, membrane receptors (CD14 and Toll-like receptor 4) and intracellular signaling amplification machinery. LBP promotes rapid binding of LPS and forms aggregates that bind to mCD14 [39]. Membrane CD14 is important for conferring sensitive cellular responses to LPS. LPS induction of cytokine release, particularly TNF-α and IL-1, is probably the central most important event in initiating the acute-phase response to infection [3]. LPS stimulates the cells by ligating specific membrane receptors like CD14 that are important for cytokine release. Anti-CD14 mAb blocks release of TNF-α from macrophages [6, 18, 39] and protects primates from endotoxin shock [9, 35].

Bovine coliform mastitis is an inflammation of the mammary gland caused by Gram- negative bacteria, where *Escherichia coli* is the most common pathogen [10, 19]. About 40–50% of all clinical cases of mastitis are caused by coliform bacteria [27]. Mastitis results in decreased milk production, increased veterinary costs, and culling and death of animals. Coliform mastitis is the most prevalent form of clinical mastitis, with infection by *Escherichia coli* being the most frequent. Approximately 80% of all intramammary infections by coliform bacteria will result in clinical mastitis, and about 25% of the cows will develop acute clinical symptoms [11, 12]. Coliforms are present in the cow's environment and they cannot be eradicated on a practical basis. Conventional herd management practices such as pre- and post-milking teat dipping, and dry cow antibiotic therapy have been unable to reduce the incidence of new intramammary infections by coliforms. Even in well-managed herds coliform mastitis continues to exist as an animal health problem. In addition, antibiotic treatment, extensive fluid supplementation and metabolic support are not effective in relieving symptoms associated with clinical coliform mastitis [27]. Anti-bovine CD14 mAb may provide a means of reducing clinical symptoms during acute coliform mastitis by blocking the binding of LPS to mCD14 on monocytes and macrophages, thus down-regulating release of inflammatory mediators like TNF-α and IL-1. The objectives of this study were to: (1) produce anti-rbosCD14 mAb that could be used to reduce release of inflammatory cytokines from bovine macrophages, and (2) develop an ELISA for quantitating bovine sCD14.

2. MATERIALS AND METHODS

2.1. Production of rbosCD14

The rbosCD14 was produced in a baculovirus expression system as previously described [38]. Briefly, rbosCD14, with a deletion of 14 amino acids at the C-terminal end, was generated by insect sf/9 cells infected with recombinant virus containing the gene. The rbosCD14 was purified from culture supernatant using Ni-NTA superflow agarose beads (Qiagen, Valencia, CA, USA) and FPLC system (Amersham Pharmacia, Uppsala, Sweden) with a typical yield of 4–6 mg/L of culture supernatant. Functional analysis of the rbosCD14 showed

that it reduced mortality in mice from endotoxin shock and reduced severity of intramammary infection in mice and cows after experimental challenge with *E. coli* [24, 25].

2.2. Hybridoma production and mAb purification

Four female BALB/c mice (National Cancer Center, Fredrick, MD, USA) were immunized intraperitoneally biweekly with 50 μg of rbosCD14 emulsified in Freund's adjuvant (Sigma, St. Louis, MO, USA). Six weeks after the second injection and three days prior to fusion, mice were boosted by intravenous injection of 25 μg of rbosCD14 in 0.5 mL of 0.01 M phosphate buffered 0.85% saline, pH 7.4 (PBS). Mice producing high serum antibody titers to rbosCD14 were selected by ELISA, and their spleen lymphocytes were fused with mouse myeloma SP 2/0 cells (ATCC, Rockville, MD, USA). Hybridomas were selected in medium supplemented with hypoxanthine, aminopterin, and thymidine (Sigma), and supernatants screened by ELISA (see below). Hybridomas secreting rbosCD14 antibodies were cloned by limiting dilution using mouse thymus feeder cells. Ascites were produced in female BALB/c mice following intraperitoneal injection with 0.5 mL of 2, 6, 14, 20-tetramethypentadecane (Sigma). Use of animals for this investigation was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

2.3. Polyclonal antibody production

The immunization protocol is described in detail by Fretterer and Barfield [13]. Briefly, a New Zealand white female rabbit, 4–5 pounds and specific pathogen-free, was purchased from Covance Research Products (Denver, PA, USA). The ImmuMax SR adjuvant system, an immunostimulatory biomolecule, surfactant and oil (Zonagen Inc., The Woodlands, TX, USA), was used to form an emulsion containing 15 μg of rbosCD14. The mixture of gel, rbosCD14 and ImmuMax SR chitosan was brought to pH 7.0 and emulsified with the surfactant and oil immediately before being injected subcutaneously into the scapular arch of the rabbit. Total volume of the injection was 1.0 mL and was spread over 3–4 injection sites. On day 30 post-immunization, this procedure was repeated. Before immunization, rabbits were bled from the central auricular artery to obtain baseline control sera. At 37 days post-immunization, a second blood sample was obtained to determine positive antibody response and titer.

2.4. Isotyping of mAb

A mouse hybridoma isotyping ELISA kit (Biomeda, Foster City, CA, USA) was used to isotype the mAb. Using 96-well microtiter plates, 50 μL of antigen solution was added to each well and incubated for 3 h at 37 °C. The plates were washed once with PBS, followed by addition of 200 μL of 1% bovine serum albumin (BSA) blocking solution to each well and incubated for 1 h at 37 °C. Hybridoma supernatant (100 μL) was added to each well and 50 μL of class- and subclass specific anti-mouse antibodies was added to the plates and incubated for 30 min at 37 °C. After washing the plates 3 × with PBS, 50 μL of rabbit anti-goat IgG conjugated to horseradish peroxidase (Bio-Rad) was added to each well and incubated for 30 min at 37 °C. After washing the plate 3 × with PBS, 100 μL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) chromogen/substrate solution (Sigma) was added to each well. The reaction was stopped by adding 50 μL of 2 N sulfuric acid, yielding a yellow color. Absorbance was measured using a microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at a wavelength of 450 nm.

2.5. Screening for production of anti-rbosCD14

Flat bottom 96-well microtiter plates (Dynex Technologies Inc., Chantilly, VA, USA) were coated with 100 μL of rbosCD14

Table I. Immunoglobulin isotype and results from ELISA and western blot analysis for the ten mAb developed in this study.

Mab	Isotype	ELISA		Western blot	
		bosCD14	Control	bosCD14	Control
M1-54-1	IgG1,K	+++	–	+++	–
M1-34-2	IgG2b,K	+++	–	++	–
M1-6-6	IgG2b,K	+++	–	+++	–
M3-3-8	IgM,K	+++	–	+	–
M1-34-9	IgG2b,K	+++	–	++	–
M2-7-7	IgG3,K	+++	–	+++	–
M1-54-2	IgG1,K	+++	–	+++	–
M4-2-3	IgM,K	+++	–	+++	–
M5-29-3	IgM,K	+++	–	+++	–
M5-2-8	IgM,K	+++	–	+	–

+: weak reactivity, ++: moderate reactivity, +++: strong reactivity.

(10 µg/mL in 0.1 M carbonated-bicarbonate buffer pH 9.6) and incubated at 4 °C for 16 h. The plates were washed 3 × with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Each well was blocked with 200 µL of PBS containing 1% (w/v) BSA (Sigma) for 1 h at room temperature and washed 3 × with PBS-T. Hybridoma supernatants (100 µL) were added and incubated for one hour at room temperature, and washed 3 × with PBS-T. One-hundred microlitres of HRP-conjugated goat anti-mouse IgG (H+L; Sigma) diluted 1:2000 in PBS-0.1% BSA was added, and plates incubated for 1 h at room temperature. The plates were washed 3 × with PBS and then 100 µL of 0.01% (w/v) TMB in 0.05 M phosphate – citrate buffer, pH 5.0 was added and incubated at room temperature for 15 min. The reaction was stopped with 50 µL of 2 N sulfuric acid. Color change was measured by an automated microtiter plate reader (Bio-Tek Instruments) at a wavelength of 405 nm.

2.6. Preparation of milk whey and blood plasma

Milk was obtained from five cows at intervals over a 72 h period after intramammary injection of 10 µg of lipopolysaccharide (LPS, *E. coli* 0111:B4, Sigma) LPS (10 µg) dissolved in 10 mL of 0.85 % saline and sterile filtered through a 0.22 µm cellulose acetate filter (Corning Incorporated, Corning, NY, USA). The right or left front mammary quarters were infused with either 10 mL of saline alone or LPS.

For the preparation of whey, milk samples were centrifuged at 44 000 × *g* at 4 °C for 30 min, and the fat layer was removed with a spatula. The skimmed milk was centrifuged again for 30 min as above, and the translucent supernatant collected and stored at –70 °C in aliquotes. Blood samples were obtained from the tail vein and collected in glass tubes containing K_2EDTA (Becton-Dickinson Corp, Franklin Lakes, NJ, USA), centrifuged at 1 500 × *g* for 15 min, and the clear plasma supernatant was collected and stored at –70 °C in aliquotes.

2.7. Sandwich ELISA for sCD14 in cow milk after challenge with LPS

A sandwich ELISA was used to quantify sCD14 levels in milk whey. Flat-bottom 96-well plates were coated with 5 µg/mL of mouse anti-rbos CD14 mAb M1-54-2 diluted in 0.05 M sodium carbonate, pH 9.6 at 4 °C. This mAb was selected because it gave an intensely stained band when used in the western blot (Tab. I). The plates were washed 4 × with 0.05% Tween 20 diluted

in 50 mM Tris buffered saline (TBS), pH 8.0, and subsequently blocked with 2% fish skin gelatin (Sigma) for 1 h at room temperature. Plates were washed and 100 µL of undiluted whey samples were added to each well. Rabbit anti-bovine CD14 polyclonal antibody was diluted 1:2000 in TBS buffer containing 0.2% gelatin, and 100 µL was added to each well and subsequently washed as above. One-hundred microlitres of HRP-conjugated goat anti-rabbit IgG (H+L; Promega) diluted in PBS containing 0.2% gelatin (1:5000) was added to each well. Plates were incubated for 1 h at room temperature, washed as above, and 100 µL of TMB substrate solution added to each well. The reaction was stopped by the addition of 50 µL of 2 N H_2SO_4 and the absorbance read at 450 nm on a microplate reader (Bio-Tek Instruments, Inc.). Concentrations of sCD14 were calculated by reference to a standard curve obtained by incorporating a series of concentrations of rbosCD14 in the assay.

2.8. Isolation of macrophages and monocytes and cultivation in vitro

Bovine mammary gland macrophages were obtained by daily infusion of 50 mL of sterile isotonic saline solution into the involuted mammary quarter of three normal adult cows for five consecutive days [7]. To avoid infection of the gland, the teat orifice was washed with 70% ethanol, and a sterile teat cannula was used to infuse the saline. Following gentle massage, the suspension was milked out into a 50 mL plastic conical centrifuge tube that contained 25 mL of ice cold PBS. The samples were kept on ice and transported to the laboratory. The suspension was centrifuged at 250 × *g* for 15 min at 4 °C. The cell pellet was suspended in 10 mL of PBS. Duplicate films were made on glass slides and stained with Wright stain using an automatic slide stainer. Leukocyte differential counts of 200 cells were performed on each slide at a magnification of 1250 using the battlement procedure [21]. Because large lymphocytes in cows may resemble macrophages/monocytes [16], only cells with characteristic amoeboid nucleus and vacuolated moderate blue cytoplasm with or without fine azuorphilic granules were counted as macrophages [21]. On average, 65% of the cells were macrophages with a viability, as determined by exclusion of Trypan blue, of 98%.

Cross-reactivity of the anti-rbosCD14 mAb was performed using mouse macrophages, and porcine and equine monocytes. For the preparation of mouse macrophages, primary cultures of mouse fibroblast/macrophages (MEF/Mac) were initiated by explant culture of day-16 to -18 gestation fetal mice after removal of viscera [14]. Finely minced fetal mouse tissue was plated in T25 flasks in 2 mL of Dulbecco's modified Eagle's medium with 4.5 g/L glucose (high glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 U/mL penicillin/streptomycin (10% DMEM/H). After attachment and initial outgrowth (~ 96 h), 4 mL of additional 10% DMEM/H was added to each primary culture flask. Secondary cultures were produced by washing the primary cultures twice with PBS and once with trypsin-EDTA solution. The released cells were resuspended in 10% DMEM/H and replated in T25 flasks at a 1:3 split ratio. Secondary MEF/Mac cultures were routinely passaged at a 1:4 split ratio at 7-day intervals until senescence occurred at approximately passage 12. A culture medium regimen of 3 days on 10% DMEM/H followed by 4 days on 10% DMEM/low glucose was maintained over each passage to foster maximum growth of the macrophages. By passage 3–4 approximately 70% of the cells in the cultures were macrophages.

Monocytes isolated from porcine blood were kindly provided by Dr Joan Lunney (Animal Parasitic Diseases Laboratory, USDA-ARS, Beltsville, MD, USA). Monocytes isolated from equine blood were kindly provided by Dr Martin Furr (Department of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA).

2.9. Immunoblot analysis

Macrophages isolated from involuted mammary glands were lysed in 2% lysis buffer as previously described [37]. Nuclei were removed by centrifugation at 31 000 × *g* for 20 min at 4 °C. The macrophage lysate, milk whey and serum were precleared with 50 µL of protein G-Sepharose (Pharmacia, Uppsala, Sweden) for 1 h at 4 °C with gentle rocking. The antigens were immunoprecipitated by incubating with anti-rbosCD14 mAb M1-6-6 and M5-2-9-3, or normal mouse serum and with protein G-Sepharose. The immune complexes and proteins that nonspecifically bound to protein G-Sepharose were eluted from protein G-Sepharose by heating at 100 °C for 3 min in the loading buffer without 2-mercaptoethanol. The rbosCD14 was resuspended in SDS-PAGE sample buffer, heated and resolved on a 4–12% Bis-Tris gel (Invitrogen). Separated proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) using the Mini-Protean II transfer chamber (Bio-rad). The membrane was blocked overnight at 4 °C in PBS containing 1% BSA, washed 2 × with PBS-T and sequentially incubated at room temperature with supernatant of rbosCD14 mAb for 1 h and goat anti-mouse IgG peroxidase antibody (1:2000 in PBS-1% BSA) for 30 min. The membrane was washed 5× with PBS-T, 5× with distilled water and developed using Sigma Fast DAB peroxidase substrate (Sigma).

2.10. Flow cytometric analysis

One-million cells were reacted with anti-rbosCD14 mAb (1:100), and incubated at 4 °C for 30 min. The cells were washed 3 × with PBS. Fluorescein isothiocyanate (FITC)-labeled affinity-purified antibody to mouse IgG+IgM (H+L) (KPL, Gaithersburg, MD, USA) was added at a 1:100 dilution in PBS to cells reacted with the mAb and control cells that were not reacted with mAb. After 30 min of incubation at 4 °C, the wells were washed 3 × with PBS and resuspended in 200 µL PBS. Flow cytometric analysis was performed using a Coulter Epics Profile I-Argon laser flow cytometer (Coulter Electronics Inc., Hialeah, FL, USA). The laser was set at 488 nm wavelength, 7.0 to 7.5 A current and 15 mW power; it was aligned by use of fluorospheres (Immuno-Check, Epics alignment fluorospheres, Epics Division, Coulter Corp., Hialeah, FL, USA). Gains for forward-angle light scatter were set at 10, and logarithmic transformations were used for side (90°) light scatter and for green fluorescence. Locations of the cell populations in dot plots had been determined previously on the basis of analyses of pure populations of leukocytes [28]. A bitmap (electronically defined observation area) was drawn around the large mononuclear cell population that contained both large lymphocytes and macrophages, and fibroblasts for the mouse macrophage preparation. Percentage of cells fluorescing (% F) and the level of expression of CD14 molecules (log mean fluorescent channel [LMFC]) were recorded by measuring the green fluorescence associated with the gated mononuclear cell population.

2.11. Use of anti-rbosCD14 to block in vitro release of TNF-α from cultured monocytes

Blood was collected from the median caudal vein of 5 cows in 15-mL evacuated tubes containing 1.5 mL of acid citrate dextrose (40% trisodium citrate, 14.5% citric acid, 45.5% dextrose). For the isolation of monocytes, the blood was centrifuged at 900 × *g* for 30 min at 4 °C with the brake disengaged. The buffy coat layer was removed, and transferred into a sterile 40 mL glass vial containing 20 mL Hanks' balanced salt solution (HBSS, Invitrogen Corp., Grand Island, NY, USA). Ficoll-paque (Amersham Pharmacia, Biotech, AB, Uppsala, Sweden) (12.5 mL) was layered over the HBSS in a 50 mL centrifuge tube. After centrifuging for 30 min at 450 × *g*, the band containing the mononuclear cells was removed and transferred into a 50 mL centrifuge tube, resuspended in

45 mL of HBSS and centrifuged at 180 × *g* for 10 min at 5 °C. Contaminating red blood cells were removed by resuspending the cells in 5 mL HBSS, 10 mL of double distilled water was added, gently mixed for 18 s and then 10 mL of 2 × minimun essential medium Eagle (Sigma) was added to restore isotensitity. The cell suspension was centrifuged 180 × *g* for 10 min at 5 °C and resuspended in 10 mL HBSS.

A 1 mL aliquot of the cell suspension containing 10^5 monocytes was added to 24 well tissue culture plates and incubated for 1 h at 37 °C. After incubation, the nonadherent cells were removed and then 1 mL of medium RPMI 1630 (Gibco Brl, Life Technologies, Grand Island, NY, USA) containing penicillin (100 U/mL), streptomycin (50 μg/mL) (Sigma) and 10% heat-inactivated fetal bovine serum was added. Monocytes were preincubated with 10 μg of anti-rbosCD14 mAb (M1-54-1) or media for 20 min at 4 °C. Next, LPS (0.1 ηg, 0.01 ηg, 0.001 ηg) was added to the wells and incubated for 24 h at 37 °C in 5% CO_2. The media from each well was collected and centrifuged at 9 300 × *g* for 30 min. The supernatant was collected and assayed for TNF-α as previously described [29].

2.12. Statistical methods

A t-test or analysis of variance with the Tukey post hoc comparison test was used to compare the mean responses between a single experimental group and its control or among multiple experimental groups, respectively. All statistical analyses were performed using GraphPad InStat version 3.05 for Windows (GraphPadSoftware Inc., San Diego, CA, USA). A *P*-value of 0.05 or less was considered significant.

3. RESULTS

3.1. Production of anti-rbosCD14 mAb

Following immunization with the rbosCD14, 10 murine hybridomas secreting mAb reactive with the immunogen were identified. Table I summarizes the immunoglobulin isotypes, ELISA and western blot reactive assay results. All antibodies were reactive with rbosCD14, but not with BSA, cell culture media, or with non-immunized mouse serum that were used as negative controls. Representative ELISA results using the strongly reactive mAb M 1-6-6 are illustrated in Figure 1.

3.2. Intramammary challenge with LPS increases concentration of sCD14 in milk.

To determine whether the mAb could be used to detect and quantify sCD14 in whey a sandwich ELISA was used to quantitate sCD14 in milk (Fig. 2). Mammary gland quarters receiving saline alone showed no change in sCD14 levels throughout the study. In contrast, significant ($P < 0.05$) increases in sCD14 were evident within 8 h of LPS infusion and this increase persisted for an additional 40 h. Maximal levels of sCD14 were observed 24 h after LPS challenge.

3.3. Western blotting

By western blot analysis, all mAb reacted with a 40 kDa protein for rbosCD14 (Fig. 3). The mAb also recognized 53 kDa and 58 kDa sCD14 in bovine milk and bovine plasma (Fig. 3). Four additional mAb (M 5-29-3, M 4-2-3, M 3-3-8, M1-6-6) also strongly recognized a 47 kDa mCD14 protein and a possible accessory 24 kDa protein from a lysate of mammary macrophages (Fig. 4). However, two monoclonal antibodies (M 2-7-2, M 1-54-2) strongly reacted with the 40 kDa recombinant protein but not to the macrophage lysate. Additionally, four monoclonal antibodies (M 1-34-9, M 1-52-8, M 3-9-1) showed only one 47 kDa band from the macrophage lysate (data not shown).

3.4. The binding of mAb to macrophages and cross-reactivity studies

To study binding of anti-rbosCD14 mAb to mCD14 on macrophages from mammary

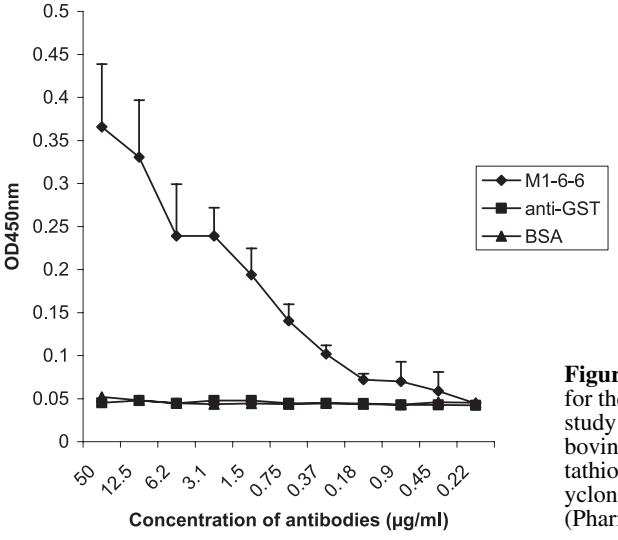

Figure 1. Representative ELISA results for the anti-rbosCD14 developed in this study for mAb M1-6-6. Controls include bovine serum albumin (BSA) and glutathione-S-transferase (anti-GST) a polyclonal antibody purified from goat sera (Pharmacia).

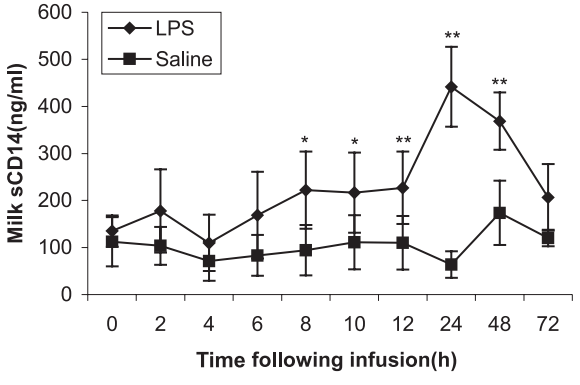

Figure 2. A sandwitch ELISA was used to quantitify sCD14 in milk obtained from mammary quarters of five cows that received either saline or LPS (10 µg). Mean (± SE). Significantly increased compared to saline control at the same time point (* $P < 0.05$, ** $P < 0.01$).

glands of healthy cows and to perform cross-reactivity studies, flow cytometry was performed using anti-mouse rbosCD14 mAb. The reactions of the 10 anti-rbosCD14 mAb (diluted 1:1000) with macrophages isolated from involuted bovine mammary glands as measured by flow cytometry is shown (Tab. II). After correcting for autofluorescence of the cell preparation (9.9%), the percentage of cells recognized by the 10 mAb ranged from 58.0% to 66.9%. The log mean fluorescent channel (LMFC) ranged from 3.0 to 4.1. No correction for autofluorescence was applied because with a low percentage of the cells fluorescing, the LMFC will be widely scattered along the X axis and will cause inflated values for LMFC. MAb M1-6-6 and M3-3-8 showed the highest binding to macrophages from involuted mammary secretions and bound to 67% (LMFC 16) of cells in a mouse macrophage cell culture. In contrast, these mAb did not bind to blood monocytes isolated from swine and horse blood (data not shown).

3.5. TNF-α release from monocytes incubated with anti-rbosCD14 mAb and exposed to LPS

Inhibitory effect of anti-rbosCD14 mAb (M1-54-1) on the LPS induced production

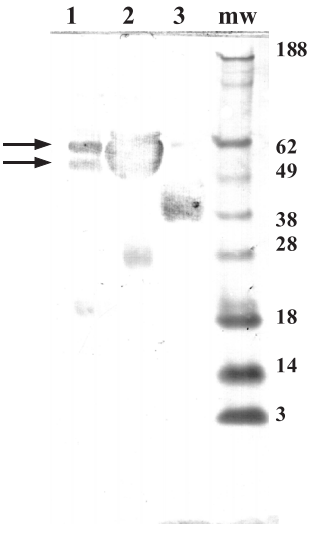

Figure 3. Representative western blot using mAb M5-29-3 showing identification of 53 and 58 kDa proteins, indicated by arrows, in milk whey (lane 1) in blood plasma (lane 2) and a 40 kDa protein in rbosCD14 (lane 3). Milk whey was prepared eight hours after intramammary injection of 100 μg of LPS.

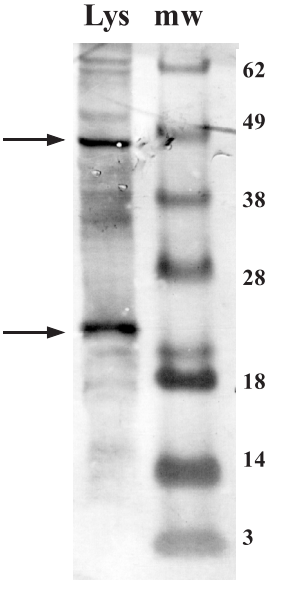

Figure 4. Representative western blot of a macrophage lysate (Lys) using anti-rbosCD14 mAb M1-6-6 showing identification of 47 and 24 kDa proteins (arrows).

of TNF-α by adherent monocytes during 24 h of incubation was dose dependent (Fig. 5). The inhibition of TNF-α by anti-rbosCD14 mAb was obtained at all concentrations of LPS (1 to 100 ηg/mL, $P < 0.05$). The greatest inhibition (61%) of TNF-α production was achieved when 100 ηg/mL of LPS was used to stimulate the monocytes. With 1 and 10 ηg of LPS/mL less inhibition (37% and 54%) was observed. The anti-rbosCD14 mAb did not affect the production of TNF-α by unstimulated monocytes (data not shown).

4. DISCUSSION

Several of the mouse anti-rbosCD14 mAb developed in this study showed strong reactivity with bovine mammary macrophages by flow cytometry and to sCD14 in milk whey when used in an ELISA. Using the anti-rbosCD14 mAb, we demonstrated that the relative molecular mass for mCD14 from the bovine macrophage lysate was 47 kDa. Bovine mCD14 is shorter than human mCD14, which has a molecular mass of 50–58 kDa. Also, the ten murine anti-rbosCD14 mAb developed in this study show different recognition patterns to macrophages by also recognizing a 24 kDa band. This band may be a mCD14 precursor or a truncated mCD14 degradation product. We also demonstrated that sCD14 in bovine milk and blood exists in two forms of 53 and 58 kDa. It was previously shown that monocyte and macrophage mCD14 exists in two soluble forms in normal human plasma [8]. These two sCD14, which are referred to as sCD14 α (low Mr) and sCD14 β (high Mr), can also be synthesized and released into plasma by normal human monocytes. Different molecular mass forms of sCD14 are present in human serum depending on its origin [5, 8, 22]. The lower molecular mass forms are derived from the proteolytic cleavage of monocyte mCD14, and, therefore, have a truncated C-terminus without the GPI anchor. These same cells also produce a high molecular mass CD14 receptor lacking the GPI

anchor, which is directly exocytosed. Varying degrees of glycosylation also contribute to the heterogeneity of the sCD14 molecule [32].

Table II. Reactivity of murine anti-rbosCD14 mAb with macrophages isolated from involuted bovine mammary glands (n = 2 cows) as measured by flow cytometry. Specific mAb binding was detected by FITC-labeled goat-anti-mouse IgG and IgM.

mAb	Percentage cells fluorescing[a,b]	Log mean fluorescent channel[b,c]
M1-54-1	67.4 ± 3.8	3.2 ± 0.7
M1-34-2	68.8 ± 2.1	3.0 ± 0.5
M1-6-6	74.9 ± 8.7	3.7 ± 0.4
M3-3-8	76.3 ± 2.5	4.1 ± 0.5
M1-34-9	68.0 ± 4.3	3.2 ± 0.7
M2-7-7	71.8 ± 4.4	3.4 ± 0.8
M1-54-2	71.6 ± 3.1	3.3 ± 0.6
M4-2-3	73.0 ± 8.0	3.4 ± 0.6
M5-29-3	72.1 ± 6.5	3.5 ± 0.8
M5-2-8	68.9 ± 7.9	3.3 ± 0.8
Control[d]	9.4 ± 3.8	1.9 ± 0.1

[a] The cell preparation averaged 65% macrophages.
[b] Mean ± standard error of the mean.
[c] A measure of CD14 receptor density of the cell surface.
[d] Isolated cell preparation incubated with FITC-labeled goat anti-mouse IgG and IgM only.

Using the anti-rbosCD14 and ELISA that was developed in this study, we were able to demonstrate an increase in sCD14 in milk whey after intramammary injection of LPS. The increase paralleled previously reported increases in sCD14 in milk whey in response to LPS using commercially available mAb [3]. In that study, it was also shown that increases in milk neutrophil counts preceded the increase in sCD14. This suggests that neutrophils may be the source of the sCD14, because it was previously shown that neutrophils express sCD14 on their cell surface [28].

Effective approaches for the treatment of Gram-negative infections lag behind the substantive advances made in understanding host mechanisms involved in response to sepsis. This makes the relationship between the timing of delivery and the efficacy of potential therapeutics for septic shock a critical issue. In the present study, LPS induced release of TNF-α by isolated monocytes was reduced by anti-rbosCD14 mAb. The inhibition of TNF-α secretion by anti-rbosCD14 mAb can be explained by reduced activation of monocytes during interaction of these stimuli with CD14. The inhibitory effect of anti-rbosCD14 mAb on the secretion of TNF-α by LPS stimulated monocytes can be due to impaired binding of these stimuli to CD14 and/or decreased activation of monocytes via CD14. The region of CD14 that

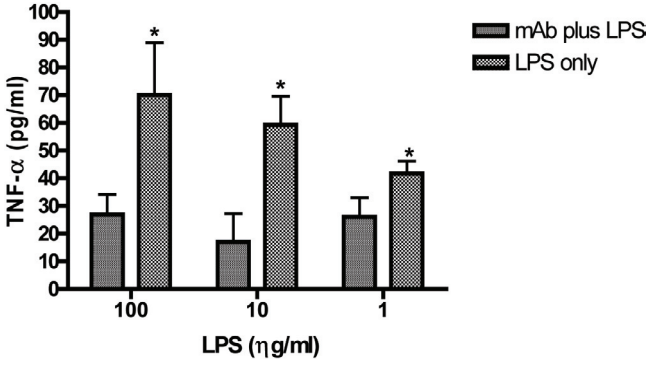

Figure 5. Anti-rbosCD14 mAb inhibits TNF-α response of monocytes after stimulation with LPS. Monocytes were preincubated with anti-rbosCD14 for 1 h before addition of LPS. Data are presented as the mean ± standard error of the mean (n = 5 cows). * $P < 0.05$.

recognizes and binds LPS has been determined [31, 32, 36], and most likely Gram-negative bacteria bind via LPS at their surface to the same site of CD14. Intact Gram-negative bacteria can bind to m- and sCD14 in the presence of serum [33], which indicates that LPS incorporated into the membrane of Gram negative bacteria can interact with CD14. Intact Gram-negative or Gram-positive bacteria are more powerful stimuli for cytokine production by monocytes than are shed bacterial components [34]. Thus, it is conceivable that exudates from macrophage containing phagocytosed bacteria include cytokines that are involved in the initial clinical manifestations [34]. It can be anticipated that in vivo, blocking of the CD14 receptor will inhibit the inflammatory response to LPS and may be beneficial in severe sepsis. Indeed, anti-CD14 mAb administered after injection of LPS, protected rabbits from death and renal and pulmonary injury, and prevented hypotension and leukopenia [30]. In cynomolgus monkeys, pretreatment with anti-CD14 mAbs derived from the same murine component 28C5 as IC14 prevented LPS induced hypotension and reduced plasma cytokine levels [9]. These results support the concept that anti-CD14 treatment provides a new therapeutic window for the prevention of pathophysiologic changes that result from exposure to LPS during septic shock.

The anti-rbosCD14 mAb developed in the current study may be useful in reducing clinical symptoms associated with augmented release of TNF-α by monocytes. The mAb will afford the opportunity to relate various functional characteristics to specific groups of cells, which will lead to a better understanding of the interrelationship between CD14 and LPS.

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