Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation

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Edited by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, and approved November 16, 2000 (received for review September 19, 2000)

Induction of resting B cell growth and differentiation requires a complex series of temporally coordinated signals that are initiated on contact with activated helper T cells. These signals complement one another, each rendering the B cell susceptible to factors supporting progressive activation. Here, we demonstrate that soluble CD14 (sCD14) bypasses the physiological sequelae of events that limit B cell activation. B cell growth and differentiation *in vitro* is induced by both native and recombinant forms of sCD14 at nanomolar concentrations. sCD14-mediated cellular activation does not require membrane CD14 expression, depends on a region of CD14 that is not involved in lipopolysaccharide binding, and requires functional Toll-like receptor 4. Consistent with biological activity of sCD14 *in vitro*, its administration to neonatal mice enhances Ig secretion. The results presented establish sCD14 as a naturally occurring soluble B cell mitogen of mammalian origin.

CD14 is a glycosyl-phosphatidyl-inositol anchored membrane protein (mCD14) expressed on mature monocytes (1, 2). It functions as a coreceptor for bacterial lipopolysaccharide (LPS) and triggers the induction of inflammatory responses (3). One consequence of LPS-mediated monocyte activation is the release of soluble CD14 (sCD14) (4), and increased levels of circulating sCD14 correlate with infection and autoimmunity (5–8).

sCD14 has been postulated to desensitize monocytes through blunting their production of inflammatory cytokines in response to endotoxin (9). However, this role remains contentious, as humans respond immediately and briskly to endotoxin despite containing 1,000-fold molar excess of serum sCD14 relative to the concentration of LPS observed during sepsis (10). The present study establishes a previously unrecognized function of sCD14 and demonstrates its broader spectrum of biological activities.

Materials and Methods

Cell Preparation, Activation, and Inhibition. All splenic B cell cultures were done in serum-free medium with high buoyant density cells isolated from C57BL/6 mice as described (11). Cells (1.5×10^5) were cultured in 0.2 ml in the presence of sCD14 or LPS derived from *Salmonella typhosa* 0901. Cultures were pulsed with 1 μCi of ^{3}H-TdR at 40 h and harvested 6 h later, and thymidine uptake was assessed. For quantitation of B7.1 and B7.2, cultures of B cells were stimulated with either 50 μg/ml LPS or 0.3 μg/ml bovine lactation-associated immunotrophic protein (Bo-LAIT), harvested at 24 h, and stained with biotinylated anti-B7.1 (mAb 16.10A1, ref. 12) or anti-B7.2 (mAb GL-1, ref. 13) followed by R-phycoerythrin (PE)-conjugated streptavidin. Expression was quantitated by using a Becton Dickinson FACScan. For quantitation of secreted Ig, replicate B cell cultures containing either 50 μg/ml LPS or 0.5 μg/ml Bo-LAIT were harvested at the indicated times. Ig isotypes in culture supernatants were quantified by commercially available ELISA kits. Levels of of IgM^a in the serum of developing [BALB/c (IgM^a) $\times$ C57bl/6 (IgM^b)]F$_1$ pups were determined by ELISA using mAb b-7-6 (14) as the capture antibody followed by biotinylated anti-mouse IgM^a as the developing antibody. TEPC 183 (mouse IgM^a, κ) was used as standard. Signals were revealed by using horseradish peroxidase (HRP)-conjugated streptavidin.

Human B cells were isolated from suspensions of tonsil leukocytes. Cells were labeled with biotinylated mAb specific for CD3ε followed by avidin-conjugated "microbeads" and passed through MACS (Becton Dickinson). The effluent population contained $<1\%$ T cells and $>98\%$ B cells as assessed by immunofluorescence. B cells were cultured as described above in the presence or absence of submitogenic concentrations of plate-bound mAbs (coated at 1:1) specific for human Igκ (mAb LO-HK-3, ref. 15) and Igλ (mAb LO-HL-2, ref. 15). Cultures were pulsed at 60 h with 1 μCi of ^{3}H-TdR and harvested 12 h later, and thymidine uptake was assessed.

Inhibition of sCD14 activation of mouse B cells by CD14-specific mAbs was assessed by preincubating native human (nHu) CD14 (1 μg/ml) with the indicated concentrations of CD14-specific mAbs 3C10 (mouse anti-human, IgG$_{2b}$, ref. 16), MEM18 (mouse anti-human, IgG$_1$, ref. 17), or their isotype controls 12CA5 [mouse anti-hemagglutinin (HA), IgG$_{2b}$, ref. 18] and W3/25 (mouse anti-rat CD4, IgG$_1$, ref. 19), respectively, for 2 h at 37°C followed by the addition of 1.5×10^5 B cells. Cultures were pulsed and harvested as described above.

Induction of membrane Ig (mIg) κ expression by 70Z/3 was assessed by culturing 8×10^4 cells in 0.1 ml. After stimulation with nBo, recombinant bovine (rBo), nHuCD14, or LPS for 20 h, cells were stained with R-phycoerythrin (PE)-conjugated goat anti-mouse Igκ-specific antibody, and the proportion of Igκ^+ cells was assessed by using a Becton Dickinson FACScan. Inhibition of nHuCD14 induction of mIgκ expression on 70Z/3 cells by CD14-specific mAbs was assessed by preincubating 0.75 μg/ml nHuCD14 in 0.1 ml with mAbs 3C10 or MEM18, or their isotype controls 12CA5 and W3/25, respectively. After a 2-h incubation at 37°C, 8×10^4 70Z/3 cells/well were added, and mIgκ expression was assessed as described above.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: sCD14, soluble CD14; mCD14, membrane CD14; LPS, lipopolysaccharide; TLR, Toll-like receptor; RSDPLA, diphosphoryl lipid A from *R. sphaeroides*; PMB, polymyxin B; HRP, horseradish peroxidase; HA, hemagglutinin; LAIT, lactation-associated immunotrophic protein; Bo-LAIT, bovine LAIT; nBo, native bovine; nHu, native human; rBo, recombinant bovine; mIg, membrane Ig.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF141313).

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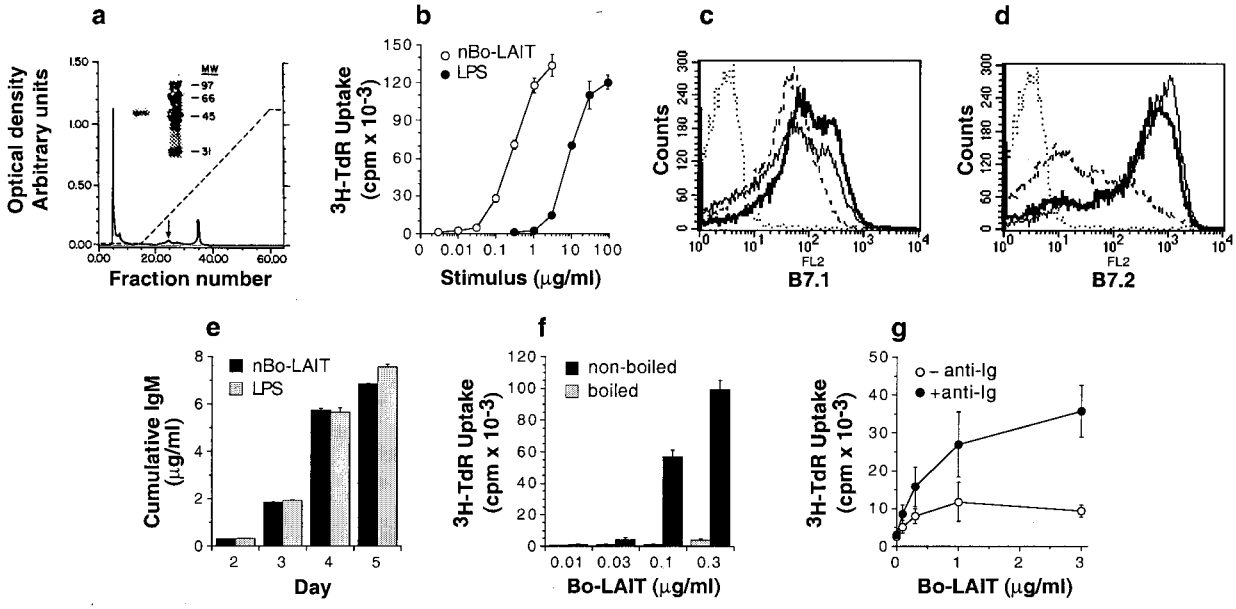

Fig. 1. Bo-LAIT stimulates the growth and differentiation of resting B cells. (*a*) The chromatograph of the last step of Bo-LAIT purification on hydroxy-apatite. Arrow indicates the peak bioactive fraction. The left track of the *Inset* depicts SDS/PAGE of 5 μg of protein derived from the peak bioactive fraction followed by silver staining. (*b*) Comparative B cell growth promoting activity of Bo-LAIT and LPS. (*c* and *d*) Bo-LAIT induced up-regulated expression of B7.1 and B7.2. Dotted line, isotype control; dashed line, unstimulated cells; solid line, 50 μg/ml LPS; bold line, 0.3 μg/ml Bo-LAIT. (*e*) Comparative differentiation promoting activity of 0.5 μg/ml Bo-LAIT and 50 μg/ml LPS. (*f*) Heat lability of nBo-LAIT. Samples were boiled or not for 10 min and used to stimulate high buoyant density murine splenic B cells. (*g*) Growth-promoting activity of nBo-LAIT on human tonsil B cells in the presence (+) or absence (−) of a submitogenic concentration of immobilized mAbs specific for human Igκ and Igλ.

Diphosphoryl lipid A from *Rhodobacter sphaeroides* (RSDPLA) inhibition was assessed by preincubating 8×10^4 70Z/3 cells in 0.1 ml with 10 μg of RSDPLA for 1 h at 37°C followed by the addition of sCD14. Cells were analyzed for Igκ^+ cells as described above. Polymyxin B (PMB)-mediated inhibition was assessed by preincubating 0.1 μg of nBoCD14, 1 μg of LPS, or 3 units/ml of IFNγ with PMB for 1 h in 0.1 ml at 37°C, followed by the addition of 8×10^4 70Z/3 cells. Expression of Igκ was assessed as described above.

Quantitation of sCD14 in Human Milk and Serum. sCD14 concentrations in milk and serum were assessed by ELISA (IBL, Gunma, Japan). Total protein was assessed by using a colorimetric detection system.

Cloning, Expression, and Isolation of rBoCD14. A cDNA library derived from bovine peripheral blood monocytes was screened with human CD14 cDNA. A 1.4-kb cDNA was isolated and sequenced. The following PCR primers were used to amplify the coding region of bovine CD14: forward, 5′-GCTAGCGCTAGCCACCATGGTGTGCGTGCCCTACCTGCTG-3′; reverse, 5′-GCTAGCGCTAGCCGCGAAGCCTCGGGCTCCTTGAAG-3. Amplified fragments were cloned into baculovirus transfer vector pETL (20) containing a DNA fragment encoding a C-terminal nonapeptide from influenza hemagglutinin (HA). The recombinant baculovirus clones were selected and purified according to established protocols (21, 22).

rBoCD14 was expressed in the mouse mammary tumor cell line C127 (American Type Culture Collection) by using the episomal vector pBPV, modified by insertion of a neomycin resistance gene (pBPVneo-13 vector). The BoCD14 coding region with HA tag was amplified from the pETL-HA vector by using the following primer set: forward, 5′-CTCGAGCTCGAGGCTAGCCACCATGGTGTGCGTGCC-3′; reverse, 5′-CTCGAGCTCGAGGGATCCCTAAGCGTAATCTGGAAC-3′. The recombinant plasmid was electroporated into C127, and drug-resistant stable transformants were selected.

rBoCD14 derived from both expression systems was affinity-purified on Sepharose 4B conjugated with mAb-3C10, mAb-12CA5, or rabbit-anti-BoCD14.

Immunoblotting. CD14 (200 ng) was resolved and transferred to poly(vinylidene difluoride) membrane, blocked with 5% milk, and developed with either rabbit anti-nBoCD14, which cross reacts with human and mouse CD14, or HA-specific mAb 12CA5 and revealed with HRP-conjugated protein A and HRP-conjugated goat anti-mouse IgG, respectively.

Results

Purification and Characterization of LAIT Protein from Bovine Colostrum. Using classical protein fractionation techniques, a 50-kDa glycoprotein was isolated from bovine colostrum based on its capacity to induce B cell growth and differentiation. This molecule originally was termed LAIT protein. Illustrated in Fig. 1*a* is the chromatograph and a silver-stained gel (*Inset*), depicting the results of the last step of the purification of Bo-LAIT to be described in detail elsewhere. Fig. 1*b* shows a comparative dose response of Bo-LAIT and LPS-mediated activation of high buoyant density murine splenic B cells. Comparable results were obtained with high buoyant density B cells further sorted by FACS to >99.8% purity (not shown). Fig. 1 *c* and *d* illustrates the capacity of Bo-LAIT to induce the up-regulated expression of B cell activation markers B7.1 and B7.2, respectively. Both Bo-LAIT and LPS preferentially stimulate the increased expression of B7.2.

Bo-LAIT also induced the differentiation of murine resting B cells to high rate Ig secretion (Fig. 1*e*). Shown are the cumulative concentrations of IgM present in culture supernatants of resting B cells stimulated with either 50 μg/ml LPS or 0.5 μg/ml Bo-LAIT for the indicated culture period. Supernatants har-

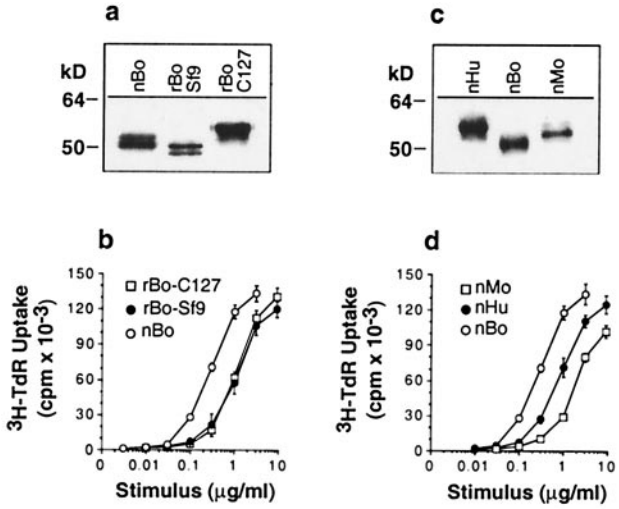

Fig. 2. Growth-promoting activity of recombinant and native sCD14. (*a*) Immunoblot of colostral nBoCD14 (200 ng) and 200 ng each of rBoCD14 derived from Sf9 and C127 expression systems, revealed with rabbit anti-nBoCD14 followed by HRP-conjugated protein A. Proteins were affinity-purified by using mouse anti-human CD14, mAb 3C10. Lane 1, molecular weight markers; lane 2, nBoCD14; lane 3, Sf9-derived rBoCD14; lane 4, C127-derived rBoCD14. (*b*) Stimulation of B cell growth by nBoCD14 and rBoCD14. (*c*) Immunoblot of nCD14 derived from bovine, mouse, and human performed as above. CD14 was isolated from sources as described in the text and affinity-purified with mouse anti-human CD14, mAb 3C10. (*d*) B cell growth-promoting activity of nCD14 derived from bovine, human, and mouse.

vested from these cultures at 24-h intervals contained comparable levels of IgM, indicating that the rates of secretion induced by the two stimuli were comparable (data not shown). Bo-LAIT reproducibly induced low, but significant, concentrations of IgG_1 (5.6 ng/ml), IgG_{2a} (400 ng/ml), IgG_{2b} (11 ng/ml), IgG_3 (75 ng/ml), and IgA (5 ng/ml) in day 5 supernatants. It remains to be determined whether this reflects bona fide isotype switching or the stimulation of the low proportion (1–5%) of IgG and IgA expressing B cells in the population. Both the growth (Fig. 1*f*) and differentiation (data not shown) promoting activities of Bo-LAIT were heat labile.

The capacity of Bo-LAIT to stimulate the growth of human B cells was assessed in the presence or absence of a 1:1 mixture of immobilized mAbs specific for human Igκ and Igλ (15) chains (Fig. 1*g*). Bo-LAIT stimulated the growth of purified human tonsil B cells, resulting in indices of stimulation at 60 h of up to 5-fold, compared with stimulation indices of 1.5- to 2-fold elicited by LPS (data not shown). Further, the combined effects of submitogenic concentrations of antibodies specific for the B cell antigen receptor and Bo-LAIT resulted in synergistic DNA synthesis, with stimulation indices of up to 20-fold (Fig. 1*g*).

LAIT Is sCD14. The molecular cloning of Bo-LAIT involved the derivation of sequence from tryptic and cyanogen bromide fragments. The sequence derived from each of five fragments aligned with human CD14 at >80% identity. Based on this result, a cDNA library was prepared from bovine peripheral blood monocytes and probed with human CD14 cDNA. This resulted in the isolation of a 1.4-kb full-length cDNA representing a homologous bovine sequence (GenBank accession no. AF141313).

rBoCD14 was expressed in both insect Sf9 cells and mammalian C127 cells and was affinity-purified from culture supernatants by using mouse anti-human CD14 mAb 3C10 (16), the reactivity of which depends on a sequence highly conserved in CD14 among species (23). Shown in Fig. 2*a* is an immunoblot of colostral nBoCD14 in comparison with rBoCD14 derived from the two expression systems. Heterogeneity of Sf9-derived rCD14 is likely caused by differential N-glycosylation, as treatment with native *N*-glycosidase F results in a single band of 37 kDa (data not shown). It is not currently known whether the differences in apparent molecular masses of the native and recombinant molecules reflect distinct glycosylation patterns and/or different sizes of the respective core proteins. As illustrated in Fig. 2*b*, both sources of rBoCD14, in isolation, stimulate DNA synthesis and differentiation to Ig secretion (data not shown) of resting murine splenic B cells with comparable efficiencies and with specific activities of roughly 30% of nBoCD14. Neither nBoCD14 nor rBoCD14 induced growth of primary murine T cells (data not shown).

The possibility that LPS contributes in some way to the B cell stimulatory activity of sCD14 was considered. Limulus assay (Chromogenix, Molndal, Sweden) of three independent preparations of nBoCD14 and rBoCD14 contained on average 2.1 pg and 0.8 pg of endotoxin per μg of CD14, respectively. Thus, B cell cultures stimulated with 1 μg/ml of native or recombinant LAIT protein contain levels of endotoxin roughly 10^6-fold lower than the suboptimal concentration of LPS required to activate resting B cells (Fig. 1*b*).

Native Murine and Human sCD14 Are B Cell Mitogens. Human CD14 was isolated from the urine of nephrotic patients by affinity chromatography as described (24). During our analysis of the distribution of CD14-specific mRNA, we discovered that some B cell hybridomas scored positive. In circumstances where both the lymphocyte and fusion partner are murine, secreted or shed CD14 would be of mouse origin. The OKT3 hybridoma (25) was found to satisfy these conditions, and native mouse CD14 was affinity-purified from culture supernatant. Shown in Fig. 2*c* is an immunoblot comparing nCD14 derived from the two species in comparison with nBoCD14. As illustrated in Fig. 2*d*, nCD14 derived from the three species stimulates DNA synthesis by resting murine B cells. The higher specific activity of nBoCD14 was reproducible and may reflect that it is derived from a physiological source in contrast to the murine and human nCD14 used herein.

Differential Inhibition of sCD14 Activity by CD14-Specific mAbs. The capacity of mAb MEM-18 to inhibit LPS-mediated monocyte activation correlates with its specificity for that sequence of mCD14 involved in LPS binding (26, 27). In contrast, mAb 3C10 does not inhibit CD14–LPS interaction; rather, it inhibits monocyte responses to LPS by binding to an epitope on CD14 postulated to be involved in signaling (23). The results presented in Fig. 3*a* demonstrate that preincubation of nHuCD14 with mAb 3C10 profoundly inhibited its capacity to activate B cells. In contrast, preincubation of nHuCD14 with mAb MEM-18, despite its capacity to interact with sCD14 (data not shown), had no effect, as did neither of the isotype control mAbs (Fig. 3*a*). Neither mAbs 3C10 nor MEM-18 inhibited LPS-induced B cell growth (data not shown).

These results distinguish the molecular interactions involved in LPS and sCD14-mediated B cell activation and suggest that mAb 3C10 mediates its inhibitory effects through masking a region on sCD14 involved in its interaction with a putative receptor expressed by B cells. However, this interpretation is complicated if B cells express mCD14 (28, 29). In these circumstances, inhibition by mAb 3C10 could be caused by its interaction with sCD14, mCD14, or both. Analysis of the pre-B cell line, 70Z/3 (30), provided insight.

70Z/3 does not express mIg (31) but can be induced to an mIg^+ state by select stimuli, including LPS and IFNγ (30, 32). Further, 70Z/3 is CD14 negative, and forced expression of mCD14 in this cell line provided evidence for the role of mCD14 as a coreceptor

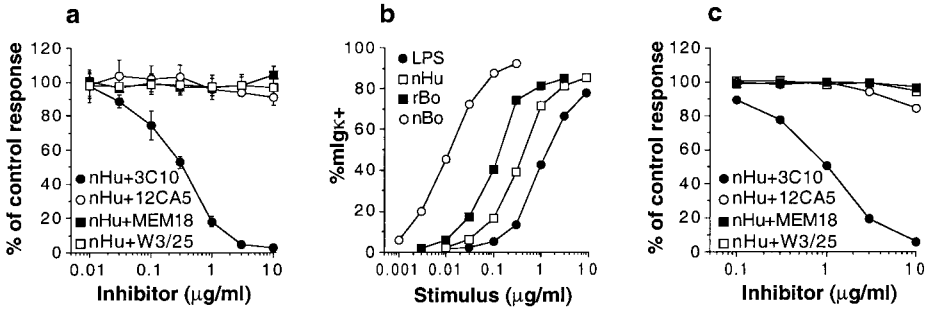

Fig. 3. Differential inhibition of sCD14-induced B cell growth and 70Z/3 differentiation by CD14-specific mAbs. (*a*) Differential inhibition of 1 μg/ml nHuCD14 induction of resting B cells by CD14-specific mAbs. Results are expressed as a percent of the response obtained in the absence of mAbs (65,125 ± 3,010 cpm). (*b*) Comparative analysis of LPS and sCD14-mediated induction of membrane Igκ expression by 70Z/3. (*c*) Differential inhibition of 0.75 μg/ml nHuCD14-mediated induction of 70Z/3 cells by CD14-specific mAbs. Results are expressed as a percent of the response induced by the stimulus in the absence of mAbs (61%).

for LPS signaling (33). We therefore determined whether 70Z/3 responded to sCD14 with the induction of mIg expression and whether this process could be differentially inhibited by mAbs 3C10 and MEM-18. As illustrated in Fig. 3*b*, nHuCD14 was roughly 5-fold more efficient than LPS in inducing the differentiation of 70Z/3, and mAb 3C10, exclusively, inhibited the function of nHuCD14 (Fig. 3*c*). Neither mAb 3C10 nor MEM-18 inhibited LPS-induced 70Z/3 differentiation (data not shown). These results formally demonstrate that the biological activity assessed in these studies depends on sCD14. They also implicate residues 7–14 of sCD14 (23), or sequences whose conformation depends on residues 7–14, in the interaction with a putative receptor on the membrane of mature primary B cells, and 70Z/3. In this regard, it has been recently reported that sCD14 binds directly to the majority of human peripheral blood B cells in a fashion readily detectable by immunofluorescence (34).

sCD14 Signals Through Toll-Like Receptor (TLR) 4. Insight into the nature of receptor(s) involved in sCD14-mediated cellular activation was derived from the observation that RSDPLA (35), a potent antagonist of LPS, blocks sCD14-mediated differentiation of 70Z/3 without affecting cell growth (Fig. 4*a*). Whereas these results indicate shared signaling pathways between sCD14 and LPS, it was important to rule out the potential role of the trace levels of contaminating LPS contained in sCD14 preparations. Toward this end, we assessed the capacity of PMB, known to inhibit endotoxin signaling through blocking the lipid A moiety (36), to inhibit sCD14 signaling. As illustrated in Fig. 4*b*, PMB specifically inhibited LPS-induced differentiation of 70Z/3, whereas differentiation induced by either sCD14 or IFNγ was unimpaired. Consistent with these results, PMB inhibited LPS but not sCD14-mediated activation of wild-type resting murine splenic B cells (data not shown).

Two TLRs have been implicated in endotoxin-mediated cellular activation, TLR2 (37) and TLR4 (38). Given the shared signaling pathways involved in LPS and sCD14 signaling, we assessed the capacity of sCD14 to stimulate the growth of B cells derived from both TLR2-deficient (37) and TLR4-deficient (38) mice. As illustrated in Fig. 4*c* and as described (37, 38), LPS signaling is profoundly impaired in the absence of TLR4, whereas the absence of TLR2 is without consequence. This is not strictly the case for sCD14-mediated B cell activation. Whereas TLR4 is essential, the absence of TLR2 consistently resulted in a modest reduction (5–20%) in sCD14-mediated responses (Fig. 4*d*).

sCD14 Is Enriched in Colostrum and Milk Throughout Lactation. The major source of native sCD14 used in these studies was bovine colostrum, harvested within 2 h of the cow having birthed. However, during the course of these studies, we also assessed the temporal expression of sCD14 in human mammary secretions. As illustrated in Fig. 5*a*, the amount of sCD14 per milligram of protein in mammary secretions from nine individuals was 100- to 1,000-fold higher than that observed in serum of the same individual. Remarkably, these enriched levels persisted up to 400 days postpartum.

These results led us to determine whether sCD14 persists in cow milk up to 300 days postpartum. Fig. 5*b* (*Inset*) shows immunoblots of nBoCD14 isolated from bovine colostrum (C) and milk (M) collected at 300 days postpartum. Whereas the basis for the apparent difference in the heterogeneity of sCD14 as visualized in SDS/PAGE is unknown, the capacity of sCD14 isolated from these two sources to stimulate the activation of resting murine B cells is comparable (Fig. 5*b*).

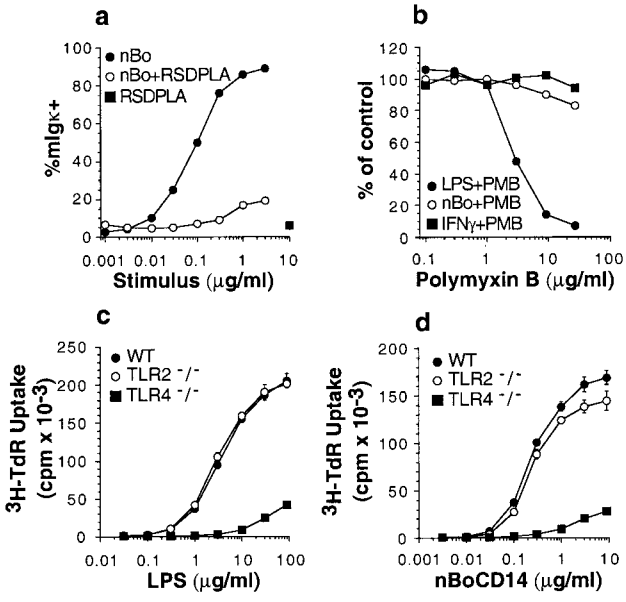

Fig. 4. sCD14 and endotoxin share signaling pathways. (*a*) RSDPLA (10 μg/ml) inhibits nBoCD14-mediated differentiation of 70Z/3. ■, 10 μg/ml RSDPLA alone. ^{3}H-TdR uptake at 24 h, subsequent to a 6-h pulse, by 1×10^5 input 70Z/3 cells stimulated with 0.3 μg/ml nBoCD14 gave rise to $1.07 \pm 0.04 \times 10^5$ cpm and $1.02 \pm 0.07 \times 10^5$ cpm in the presence and absence of 10 μg/ml RSDPLA, respectively. (*b*) PMB does not inhibit sCD14 signaling. nBoCD14 (0.1 μg), LPS (1 μg), or IFNγ (3 units/ml) were preincubated with the indicated concentration of PMB. Results are expressed as a percent of the response induced by the stimulus in the absence of PMB. Control responses induced by nBoCD14, LPS, and IFNγ were 68%, 70%, and 44%, respectively. (*c* and *d*) TLR4 protein is involved in sCD14 signaling. ^{3}H-TdR uptake by high buoyant density splenic C57BL/6 B cells derived from wild-type (WT), TLR2$^{-/-}$, and TLR4$^{-/-}$ animals stimulated with the indicated concentration of either LPS (*c*) or nBoCD14 (*d*) was assessed.

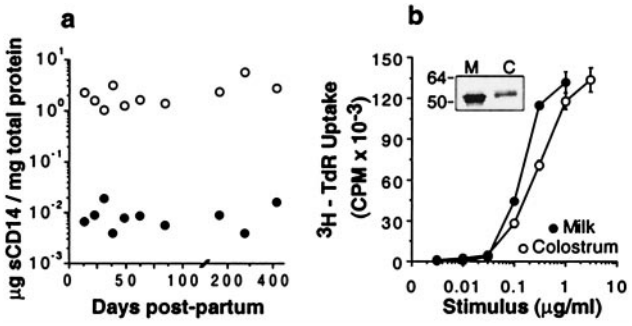

Fig. 5. Persistence of sCD14 in mammary secretions and its bioactivity. (*a*) Quantitation of sCD14 in human milk and serum from the same subject (paired symbols) at the indicated time postpartum. Indicated are the concentrations in milk (open symbols) and serum (closed symbols) in each pair of samples. (*b*) Activation of resting splenic murine B cells with sCD14 derived from bovine colostrum or milk. sCD14 was affinity-purified from either source by using mAb 3C10 and added at the indicated concentrations to B cell cultures. (*Inset*) An immunoblot of milk (M)- and colostral (C)-derived sCD14 performed as described in Fig. 2 legend.

Milk-Derived sCD14 Stimulates B Cell Differentiation *in Vivo*. To determine whether sCD14 can function in physiological circumstances, we assessed its capacity to induce B cell differentiation *in vivo*. We chose to examine the induction of B cells in neonates, as base lines are lower. Specifically, we first assessed the kinetics of paternal IgM^a allotype appearance in the serum of offspring derived from a BALB/c (IgM^a) × C57BL/6 (IgM^b) mating. The expression of IgM^a was first detected at day 2 postpartum and reached plateau levels by 30 days (Fig. 6*a*). At day 4 of life, groups of three pups were injected intraperitoneally with 10 μg of either BSA or nBoCD14, and serum was collected at the time points indicated. As illustrated in Fig. 6*b*, sCD14 induced a transient surge of B cell differentiation 4 days postinjection. The wave of enhanced IgM^a expression ultimately was overtaken by increasing levels of background IgM^a expression, and the effect of exogenous sCD14 appeared to normalize within a week (Fig. 6).

Discussion

The results presented characterize sCD14 as the first naturally occurring soluble protein of mammalian origin that in isolation induces B cell growth and differentiation. As CD14 is thought to have evolved as a receptor for endotoxin, an obvious concern throughout these studies was the potential involvement of endotoxin in supporting sCD14 activity. Converging lines of evidence discount this possibility. The trace levels of endotoxin contamination detectable by limulus assay are 10^6-fold lower than the suboptimal concentration of LPS required for resting B cell stimulation. Further, and in this context, the contaminating levels of endotoxin are 10^{-3} to 10^{-5} of the concentrations of LPS used as a costimulator of sCD14-mediated endothelial or epithelial cell activation (39, 40). The mitogenic activity of sCD14 is heat labile, inconsistent with a role for LPS that is actually boiled in aid of solubilizing. Further, the activity of sCD14 is differentially inhibited by specific mAbs. Whereas this latter line of evidence does not preclude a role for sCD14–LPS complexes, the fact that the observed activity is insensitive to PMB rules out the direct involvement of lipid A in the activation process. Importantly, in this context, the activity observed when LPS is used as a costimulator with sCD14 is sensitive to PMB inhibition (see text below and Fig. 7*b*).

Notwithstanding the plethora of reports surrounding the function of sCD14, its mitogenic properties have not been previously reported. Whereas it is not known whether any source of sCD14 will be mitogenic, the possibility that milk-derived material is unique in this regard is ruled out by the data presented. A plausible explanation is that the mitogenic activity of sCD14 depends on conformation, which in turn will be sensitive to the methods used in its isolation. Evidence in support of this notion is presented in Fig. 7*a*. Specifically, a batch of recombinant sCD14 derived from Sf9 cells was split, and equal portions were affinity-purified by using the CD14-specific mAb 3C10, a rabbit anti-nBoCD14, and mAb 12CA5, specific for the C-terminal HA-derived nonamer tag sequence. As depicted, the specific activity of these three preparations differed significantly in their capacity to stimulate mIgκ expression by 70Z/3. Importantly, the endotoxin content of these preparations did not differ significantly (0.8, 0.7, and 0.9 pg/μg sCD14 in the 3C10, 842, and 12CA5 isolated material, respectively). Hence, the measurable trace levels of endotoxin in these preparations appear not to have any functional significance. Importantly and in this context, if LPS is deliberately added to preparations of low specific activity rBoCD14, the synergy and entire response is inhibited by PMB (Fig. 7*b*).

Fig. 7*a* (*Inset*) demonstrates that any differences in the apparent molecular mass(es) of sCD14 isolated by these means are

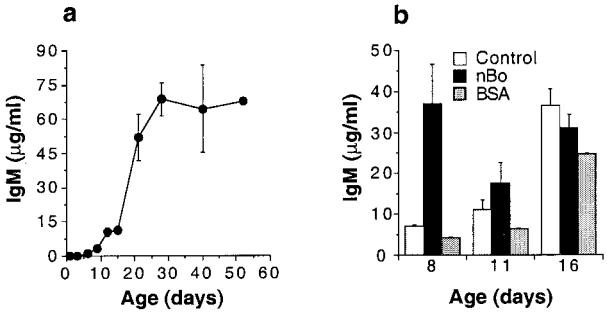

Fig. 6. sCD14 induces a B cell growth and differentiation *in vivo*. (*a*) Kinetics of appearance of IgM^a in the serum of developing [BALB/c (IgM^a) × C57bl/6 (IgM^b)]F$_1$ pups as determined by ELISA. (*b*) nBoCD14 induces a transient increase in IgM secretion *in vivo*. Groups of three [BALB/c (IgM^a) × C57bl/6 (IgM^b)]F$_1$ pups were injected intraperitoneally with 10 μg of either milk-derived nBoCD14 or BSA, or left untreated, on day 4 of life. Serum from treated and untreated animals was collected on the indicated days of life, and contained IgM^a was quantitated by ELISA as described in *Materials and Methods*.

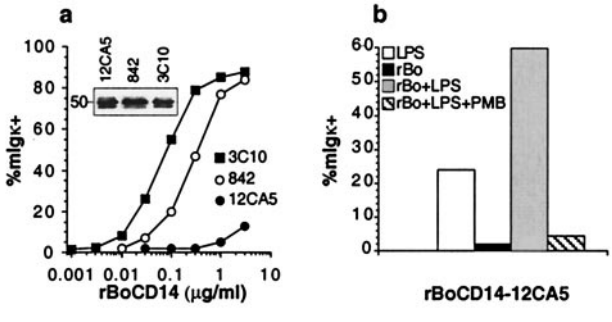

Fig. 7. (*a*) Specific activity of rBoCD14 depends on method of isolation. The indicated concentrations of rBoCD14 affinity purified by using mAbs 3C10 or 12CA5, or rabbit anti-nBoCD14 was used to stimulate Igκ expression by 70Z/3 as described in *Materials and Methods*. (*Inset*) An immunoblot of sCD14 isolated on each of the affinity columns revealed by using mAb 12CA5 followed by HRP-conjugated goat-anti-mouse IgG. (*b*) PMB inhibits LPS-mediated costimulation of low specific activity rBoCD14. rBoCD14 (0.3 μg/ml) isolated on 12CA5 was preincubated with 100 ng/ml LPS at 37°C for 1 h in serum-free medium followed by addition of 30 μg/ml PMB. After 1 h of incubation at 37°C, 70Z/3 cells were added, and Igκ expression was assessed 22 h later as described in *Materials and Methods*.

not detectable by immunoblotting in denaturing conditions. However, there is precedent for differential activity of sCD14 isoforms. Specifically, two isoforms of sCD14 have been isolated from the urine of nephrotic patients by using ion exchange chromatography, which, in isolation, differentially stimulated cytokine secretion by primary human monocytes (24). Whereas further work is required to elucidate the structural basis of these findings, they provide a rationale toward resolving the issue.

Although the involvement of other TLRs is not excluded, the results presented indicate that TLR4 is essential in conferring responsiveness to sCD14. Insights toward modeling TLR4-dependent sCD14 signaling are derived upon considering the role of CD14 in LPS-mediated activation. The current paradigm posits that Lipopolysaccharide Binding Protein complexes with LPS and facilitates the delivery of monomeric LPS to mCD14 or sCD14 (2, 41). This complex is then putatively delivered to a Toll-like signaling element that couples to intracellular signaling pathways (42). Whereas evidence supports the conclusion that LPS and TLR4 must achieve proximity to activate cells (43, 44), it remains unclear whether the triggering event involves LPS–TLR4 interaction and/or TLR4–CD14 interaction (45). The latter could depend on an interaction with LPS that confers an ''active'' conformation on CD14, enabling the CD14 interaction with TLRs (43, 44). The results presented herein demonstrate that the maintenance of an ''active'' conformation of sCD14 does not depend on the presence of physiologically relevant concentrations of LPS. Moreover, if one posits that CD14 is the trigger, it could function whether it is membrane tethered or soluble.

Based on the described enrichment of sCD14 in colostrum and milk, it is tempting to speculate on its role in actively stimulating the immune system of the suckling neonate. It may be involved in the homeostasis of IgM in the developing neonate, and its demonstrated capacity to synergize with B cell antigen receptor signaling may reflect its role in the activation of B cells before the generation of a full complement of functional helper T cells.

We thank C. Cantin for expert assistance with flowcytometry; R. Ulevitch (Scripps Research Institute, La Jolla, CA) for the human CD14cDNA; N. Qureshi (Veterans Administration Hospital, Madison, WI) for RSDPLA; and John Semple (Sunnybrook and Women's College Health Sciences Centre, Toronto, ON) for human serum and milk samples. This work was supported in part by a grant from the Medical Research Council and GEMMA Biotechnology, Inc.

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