



Ingested soluble CD14 contributes to the functional pool of circulating sCD14 in mice

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ABSTRACT

Soluble CD14 (sCD14) is a pattern recognition receptor and Toll-like co-receptor observed in human milk (5–26 µg/mL) and other bodily fluids such as blood (3 µg/mL). The most well defined role of sCD14 is to recognize lipopolysaccharide of Gram-negative bacteria and signal an immune response through Toll-like receptor 4 (TLR4). Previous research has shown ingested sCD14 to transfer from the gastrointestinal tract and into the blood stream in neonatal rats. The contribution of human milk sCD14 to circulating levels in the infant and the functionality of the protein, however, remained unknown. Using CD14^{-/-} mouse pups fostered to wild type (WT) mothers expressing sCD14 in their milk, we show herein that ingestion of sCD14 resulted in blood sCD14 levels up $0.16 \pm 0.09 \mu\text{g/mL}$. This represents almost one-third (26.7%) of the circulating sCD14 observed in WT pups fostered to WT mothers ($0.60 \pm 0.14 \mu\text{g/mL}$). We also demonstrate that ingested-sCD14 transferred to the blood remains functional in its ability to recognize lipopolysaccharide as demonstrated by a significant increase in immune response (IL-6 and TNF-α) in CD14^{-/-} pups fostered to WT mothers in comparison to control animals ($P=0.002$ and $P=0.007$, respectively). Using human intestinal cells (Caco-2), we also observed a significant decrease in sCD14 transcytosis when TLR4 was knocked down ($P<0.001$), suggesting sCD14 transfer involves TLR4. The bioavailability of human milk sCD14 established in this report confirms the importance of human milk proteins for the infant and demonstrates the need to improve infant formulas which are lacking in immune proteins such as sCD14.

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Introduction

Human milk consumption in comparison to infant formula has been linked to beneficial health outcomes for the infant such as decreased incidence of necrotizing enterocolitis (NEC) (Maayan-Metzger et al., 2004; Sullivan et al., 2010). The bioactive components of human milk linked to health benefits for infants include prebiotics, probiotics and innate immune components. One such immune component is cluster of differentiation 14 (CD14). CD14 is a pattern recognition receptor found in two forms, membrane bound CD14 (mCD14) on the surface of cells, or as a soluble protein (sCD14) in bodily fluids such as tears (0.5 µg/mL), blood (3 µg/mL) and milk (5–26 µg/mL, depending

on time of lactation) (Blais et al., 2005; Collado et al., 2012). Both forms of CD14 are responsible for the detection of several bacterial components including lipopolysaccharide (LPS) of Gram-negative bacteria (Wright et al., 1990). In combination with Toll-like receptor 4 (TLR4), detection of LPS by CD14 results in a proinflammatory immune response through both the TIRAP-MyD88 dependent pathway and the TRAM-TRIF dependent pathway (Kagan and Medzhitov, 2006; Kagan et al., 2008). sCD14, in comparison to mCD14, can also decrease the immune response to LPS by sequestering LPS from mCD14-expressing cells and providing clearance of LPS through the liver (Deng et al., 2013; Haziot et al., 2001).

To provide benefit for the infant, bioactive milk components, including sCD14, must remain intact and functional post-ingestion. In our previous study, a significant portion ($3.4 \pm 2.2\%$) of radiolabeled-human recombinant sCD14 survived gastrointestinal (GI) transit post-ingestion and was transferred, intact, into the blood of rat pups (Ward et al., 2014). The total amount of endogenous milk CD14 transferred into the blood and its functionality, however, remained unknown. We hypothesized that sCD14 is a major contributor to circulating sCD14 in infants, and that once transferred to the blood, sCD14 remains functional.

Abbreviations: CD14, cluster of differentiation 14; GI, gastrointestinal; hrCD14, human recombinant CD14; HTLV-1, human T-cell lymphotropic virus type 1; mCD14, membrane bound CD14; NEC, necrotizing enterocolitis; PP, post-partum; sCD14, soluble CD14; TLR4, Toll-like receptor 4; WT, wild type.

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Table 1

Fostering scheme used to determine the quantity and functionality of ingested sCD14 transcytosed to the blood of mice.

Pup	Foster mother	Endpoint
WT	WT	- Total circulating sCD14 - Positive control for LPS detection by sCD14
WT	CD14 ^{-/-}	- Contribution of pup-CD14 to circulating sCD14 - Impact of lack of ingested sCD14 on LPS detection
CD14 ^{-/-}	WT	- Contribution of ingested sCD14 to circulating sCD14 - Functionality of transferred sCD14 to detect LPS
CD14 ^{-/-}	CD14 ^{-/-}	- Negative control

Additionally, we sought to validate sCD14 transfer across the GI tract using human intestinal epithelial cells, and to better elucidate the mechanism by which sCD14 is transferred from the GI tract into the blood. In macrophages and dendritic cells, internalization of TLR4 has been demonstrated through a CD14 interaction (Zanoni et al., 2011). Although CD14 internalization has been shown to be independent of TLR4 in immune cells (Zanoni et al., 2011), we hypothesized that intestinal cells, which also express TLR4 (Vamadevan et al., 2010), may behave differently and that transfer of sCD14 across the epithelium may involve TLR4.

Materials and methods

Animals, fostering and LPS injection

Animal studies were conducted in accordance with the University of Ottawa's Animal Care and Veterinary Service under approval permit ID number 'BMI-129', and were approved by the University of Ottawa's Animal Care Committee. CD14 null mice (B6.129S-Cd14^{tm1Fmr}/J) and wild type controls of the same background (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Using an experimental design outlined in Table 1, mice were bred and pups were fostered at day 6 post-partum (PP) to different mothers. Starting at day 0 (pre-foster) until day 4 post-foster, pups were removed from the litter, euthanized by CO₂ inhalation, and organs were harvested (see supplemental Fig. S1 for the fostering timeline). Separately, pups were fostered as described above and injected intraperitoneally on day 2 post-foster (day 8 PP) with LPS (200 ng/g body weight, Sigma-Aldrich, Oakville, ON, Canada). Pups were monitored for signs of discomfort and at 2 h post-injection pups were euthanized by CO₂ inhalation and organs were harvested (Ward et al., 2014).

Supplementary Fig. S1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2014.03.008>.

CD14 and cytokine concentrations

To determine sCD14 concentrations in mouse milk, milk clots from the stomach of pups (15 mg) were resuspended in 150 µL of protein extraction buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 0.5 mM DTT and protease inhibitor cocktail, Sigma-Aldrich). Stomach contents and blood samples were analyzed using mouse-specific ELISAs for CD14, TNF-α and IL-6 (R&D Systems, Minneapolis, MN, USA). Stomach contents and blood samples were also analyzed by western blot to determine CD14 intactness. Samples, including 30 ng of mouse recombinant CD14 as a positive control (R&D Systems), were electrophoresed using SDS-PAGE and proteins were transferred to nitrocellulose

membranes which were blocked with 5% BSA-TBST followed by incubation with 0.5 µg/mL rabbit anti-CD14 antibody (M-305, Santa Cruz Biotechnology, Dallas, TX, USA) for one hour. Membranes were washed three times and incubated with a 1:30,000 dilution of a chicken anti-rabbit IgG horse radish peroxidase (HRP)-linked antibody (SC-2955, Santa Cruz Biotechnology). Following an additional three washes proteins were visualized using exposure to enhanced chemiluminescent (ECL) substrate (GE Healthcare, Piscataway, NJ, USA).

Caco-2 cells and transport assays

Human intestine-derived Caco-2 and HT29 cells were grown in Eagle's minimum essential media (Life Technologies, Burlington, ON, Canada) with a final concentration of 2 mM L-glutamine, 1 mM sodium pyruvate, sodium bicarbonate, 1 × Antibiotic-Antimycotic (Life Technologies) and 20% FBS (Life Technologies) at 37 °C and 5% CO₂. Biocoat HTS Caco-2 transport assays were completed as per the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells were seeded on transport membranes and differentiated for three days. Human recombinant soluble CD14 (hrCD14, R&D Systems) was added to the apical well. Media from both the apical and basal well was collected 2 h post-addition for analysis by human CD14-specific ELISA (R&D Systems). Passive transport of Lucifer Yellow (444.25 mol mass) was used to determine monolayer intactness as per the manufacturer's protocol. Lucifer Yellow (70 µM, Sigma-Aldrich) was added in addition to the soluble CD14 and was detected in the media using a Typhoon Trio Imager (GE Healthcare). Intactness of hrCD14 was determined by western blot, as described above with the exception of using an anti-human CD14 primary antibody (1:1000 dilution, R&D Systems) and a rabbit anti-mouse IgG HRP-linked secondary antibody (1:10,000 dilution, HAF007, R&D Systems).

TLR4 siRNA knockdown

Caco-2 cells and HT29 cells were grown as described above. RNA was isolated from cells using an RNeasy mini kit (Qiagen, Valencia, CA, USA) and subsequently used to create cDNA using a Maxima cDNA kit (Thermo Scientific, Waltham, MA, USA). TLR4 and GAPDH cDNA were amplified using real-time PCR conditions (ABI 7500 Real Time PCR, Applied Biosystems, Burlington, ON, Canada) as previously described and using the following primers TLR4: 5'-CTGGCTGAGACCAAGAAAGC-3', 5'-TTCAGCTCATCGATTGATAA-3' (75 bp amplicon); GAPDH: 5'-AGCCACATCGCTCAGACAC-3', 5'-GCCCAATACGACCAAATCC-3' (66 bp amplicon) (Sanchez-Munoz et al., 2011). Additionally, Caco-2 cells were seeded and differentiated on a Biocoat HTS transport assay as described above (BD Biosciences) and transfected with siRNA for TLR4 (SC-40,260) or scrambled siRNA as a control as per the manufacturer's instructions (Santa Cruz Biotechnology). After 48 h, hrCD14 (24 µg/mL, R&D Systems) was added to the apical well and transfer to the basal well was measured via ELISA, as described above. RNA was isolated from Caco-2 monolayers and analyzed by real time PCR as described above.

Immunocytochemistry and microscopy

Following transport assays, monolayers were washed twice with PBS and fixed with neutral buffered formalin. Fixed monolayers on Transwell membranes were excised from the plastic trays, hydrated for 1 h with buffer (0.1% Tween, 0.15 M NaCl, 5 mM EDTA 20 mM HEPES pH 7.5, 0.02% NaN₃) and blocked with 1% BSA in buffer for 30 min. Membranes were incubated overnight with mouse anti-CD14 antibodies (1 µg/mL, UCH-M1, Santa Cruz Biotechnology) at 4 °C, washed three times with buffer, and incubated with FITC-labeled goat anti-mouse IgG antibodies

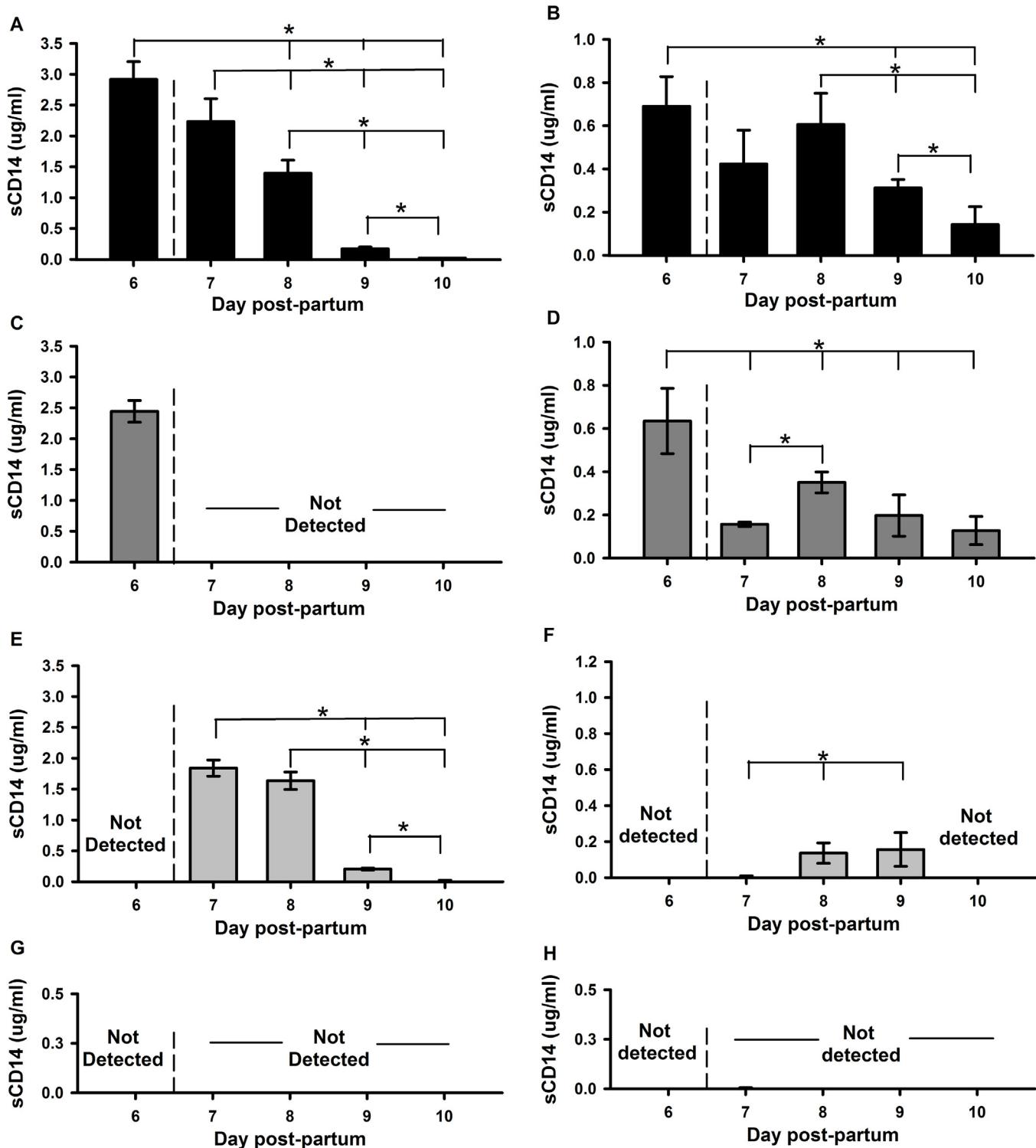


Fig. 1. Quantity of sCD14 in the stomach contents (milk clot, left panel) and blood (right panel) of mouse pups ingesting milk with or without sCD14. On day six post-partum, pups were fostered to different mothers (dotted line). (A and B), wild type pups fostered to wild type mothers; (C and D), wild type pups fostered to CD14^{-/-} mothers; E and F, CD14^{-/-} pups fostered to wild type mothers; (G and H), CD14^{-/-} pups fostered to CD14^{-/-} mothers. * $P<0.05$ by t-test, $n=3$.

(10 ng/mL, F0257, Sigma-Aldrich) for 1.5 h at room temperature. Separate membranes were incubated similarly with rabbit anti-TLR4 antibodies (1 µg/mL, SC-10741, Santa Cruz Biotechnology), followed by three washes and incubation with FITC-labeled goat anti-rabbit IgG antibody (10 ng/mL, F9887 Sigma-Aldrich). Membranes were washed three times with buffer and stained with Hoechst (10 µg/mL, Sigma-Aldrich) for 1 h at 4 °C, followed

by three more washes with buffer. Membranes were mounted on slides using ProLong Gold Anti-fade Reagent (Life Technologies) with a coverslip. Microscopy was performed using an Axio Observer D1 microscope (Carl Zeiss, Göttingen, Germany) and images were prepared using Axio Vision 4.8.2 software (Carl Zeiss). Relative signal intensities were determined using ImageJ (NIH, Bethesda, MD, USA).

Statistical analysis

Data were analyzed by 2-tailed paired or unpaired *t*-tests (Sigma Plot 12.1, Systat, San Jose, CA, USA). An alpha level of <0.05 was considered significant. In the cases where the measurands were below the level of detection (see figures, “Not detected”) values were assumed to be zero. For real time PCR data, a comparative Ct method with GAPDH as endogenous control was used, with statistical differences calculated between mean RQ-values for each experimental group.

Results

sCD14 from milk is transferred to the blood of pups

In CD14^{−/−} pups, CD14 was not detected in either the stomach contents nor blood prior to fostering the pups to different mothers (Fig. 1E and F). Once fostered to WT mothers, sCD14 was detected in the blood of CD14^{−/−} pups at day eight PP (two days post-foster) at a concentration of $0.16 \pm 0.09 \mu\text{g/mL}$, which decreased to non-detectable levels by day ten PP (4 days post-foster, $P < 0.001$, Fig. 1F). This decrease in blood sCD14 coincided with a decrease in sCD14 concentrations in the milk of WT mice, which significantly decreased from $2.23 \pm 0.37 \mu\text{g/mL}$ at day seven PP to $0.02 \pm 0.01 \mu\text{g/mL}$ at day ten PP ($P < 0.001$, Fig. 1A). The endogenous level of circulating sCD14 observed in mouse pups changed over time. In WT pups fostered to WT mothers, sCD14 was observed at $0.69 \pm 0.14 \mu\text{g/mL}$ in the blood which significantly decreased to $0.31 \pm 0.04 \mu\text{g/mL}$ by day nine PP ($P = 0.011$, Fig. 1B). In WT pups fostered to CD14^{−/−} mothers, sCD14 concentrations in the blood were similar to that of WT pups fostered to WT mothers. At day six PP, sCD14 was detected at $0.63 \pm 0.15 \mu\text{g/mL}$ in the blood of WT pups (pre-foster, Fig. 1 D). The concentration of sCD14 in the blood decreased to $0.16 \pm 0.01 \mu\text{g/mL}$ on day seven PP when fostered to CD14^{−/−} mothers ($P = 0.006$), and was similar to the amount in WT pups fostered to WT mothers by day nine PP ($0.20 \pm 0.10 \mu\text{g/mL}$, $P > 0.05$). Soluble CD14 was not detected in CD14^{−/−} pups fostered to CD14^{−/−} mothers at any time point (Fig. 1H).

A portion of the sCD14 in the stomach contents and blood of pups from all treatment groups was confirmed to be intact (48 kDa, Fig. 2). The amount of intact sCD14 in the stomach contents and blood of pups visualized by western blot also corresponded to the sCD14 concentrations obtained by ELISA (Fig. 1).

sCD14 transferred to the blood remains functional

CD14^{−/−} pups fostered to WT mothers had significantly higher IL-6 and TNF-α expression following LPS-injection (3225 ± 610 and 15.9 ± 2.7 fold increase, respectively) in comparison to CD14^{−/−} pups fostered to CD14^{−/−} mothers (693 ± 184 and 6.1 ± 1.5 -fold increase for IL-6 and TNF-α, respectively, ($P = 0.002$, $P = 0.005$) Fig. 3). The levels of sCD14 did not increase post-LPS injection in CD14^{−/−} pups irrespective of the foster mothers' genotype (Fig. 3C). WT pups fostered to WT mothers showed an increase in circulating sCD14 levels (1.6 ± 0.1 -fold increase), IL-6 levels (5333 ± 853 -fold increase) and TNF-α levels (40.3 ± 8.2) post-LPS injection (Fig. 3). WT pups fostered to CD14^{−/−} mothers showed a significantly higher increase in IL-6 expression post-LPS injection (7764 ± 946 -fold increase) in comparison to all other genotype/foster groups ($P < 0.031$, Fig. 3A). Conversely, sCD14 and TNF-α levels post-LPS injection in WT pups fostered to CD14^{−/−} mothers (2.0 ± 0.3 -fold increase and 41.7 ± 12.5 -fold increase, respectively) were not significantly different from that of WT pups fostered to WT mothers ($P > 0.05$, Fig. 3).

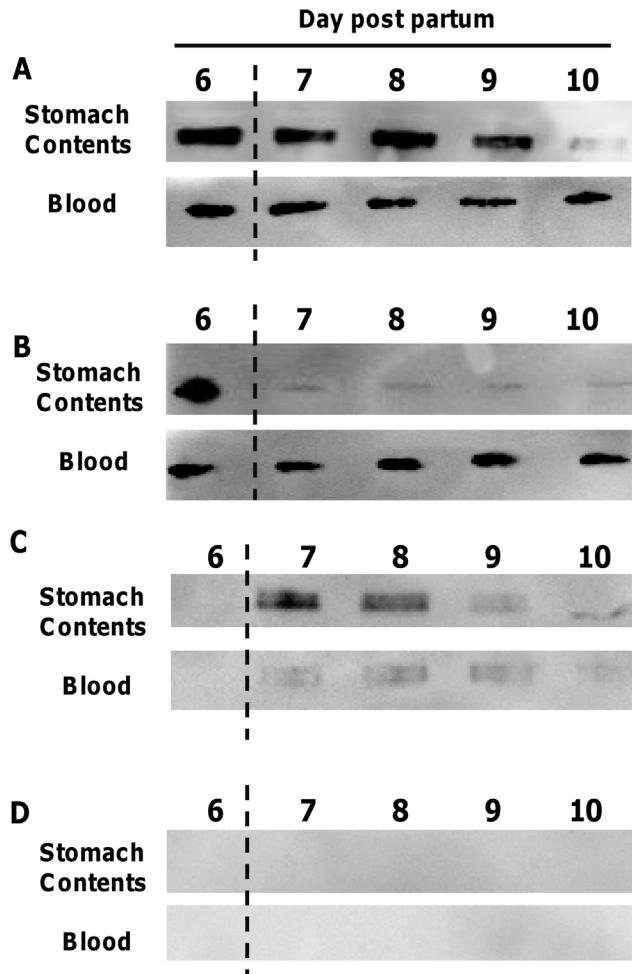


Fig. 2. Intactness of sCD14 in the stomach contents and blood of mouse pups ingest-milk with or without sCD14. (A) Wild type pups fostered to wild type mothers. (B) Wild type pups fostered to CD14^{−/−} mothers; (C) CD14^{−/−} pups fostered to wild type mothers; (D) CD14^{−/−} pups fostered to CD14^{−/−} mothers. Dotted line indicates when the pups were fostered to different mothers.

sCD14 is transferred by human cells in vitro

When added to the apical media of Caco-2 cell Transwell assays, hrCD14 was transcytosed through cell monolayers and into the basal media (Fig. 4). The amount of hrCD14 transcytosed was dependent on the initial dose in the apical well; higher initial concentrations resulted in significantly more hrCD14 observed in the basal well ($P < 0.017$, Fig. 4). After two hours of incubation, up to $3.41 \pm 0.06\%$ of the initial hrCD14 added to the apical well was transcytosed to the basal well (Fig. 4).

The integrity of the membrane was monitored by passive diffusion of Lucifer Yellow, which was minimally detected in the basal media (Supplemental Fig. S2). The intactness of pre- and post-transcytosed hrCD14 was monitored by Western blot, which showed hrCD14 from the apical and basal well to be intact (48 kDa, Fig. 4).

Supplementary Fig. S2 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2014.03.008>.

To confirm the cellular uptake of hrCD14 by Caco-2 monolayers, hrCD14 was monitored by immunocytochemistry and microscopy. The amount of hrCD14 within the Caco-2 cells was dependent on the initial apical hrCD14 concentration (Fig. 5), further confirming the ELISA results shown in Fig. 4. The production of endogenous

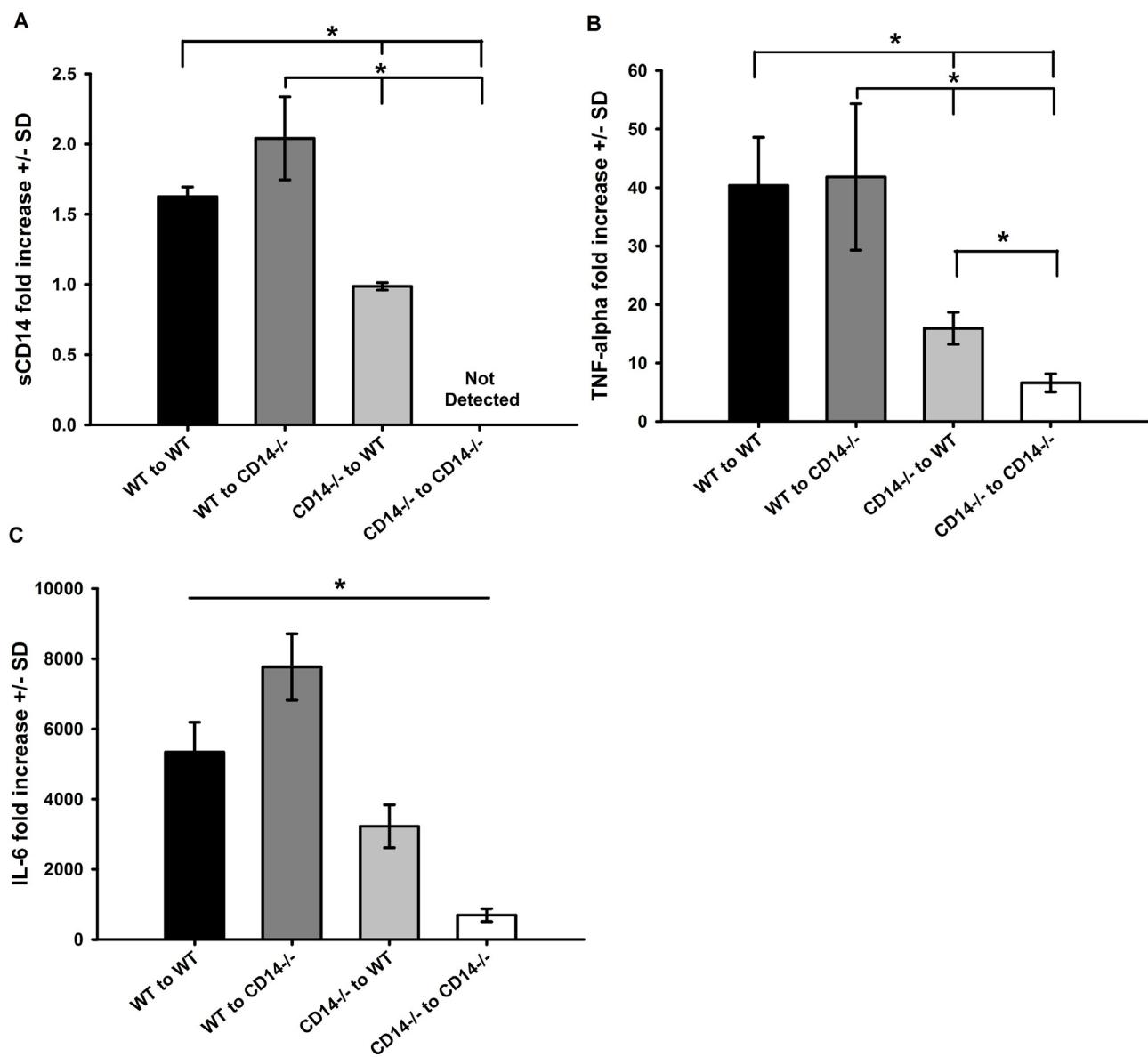


Fig. 3. Immune response to LPS following sCD14 ingestion in mouse pups, assessed via sCD14, TNF-alpha, and IL-6 levels. Pups were fostered to mothers expressing (WT) or not expressing sCD14 (CD14^{-/-}) in their milk and blood was sampled pre and post-LPS injection (200 ng/g body weight) on day two post-foster. Immune response was measured by quantifying sCD14 (A), IL-6 (B) and TNF-alpha (C) by ELISA and is expressed as a fold increase over pups fostered similarly but injected with PBS alone. *, $P < 0.05$ by t-test, $n = 3$.

CD14 (mCD14 and sCD14) by Caco-2 cells was also observed by microscopy (Fig. 5A).

sCD14 transfer by Caco-2 cells involves TLR4

TLR4 expression was observed in Caco-2 cells but not with another intestinal epithelial cell line, HT29, via real time PCR ($P < 0.001$, Fig. 6). Following TLR4-specific siRNA treatment, TLR4 expression was significantly decreased in Caco-2 cells in comparison to control siRNA (scrambled) or non-treated Caco-2 cells ($P < 0.002$, Fig. 7A). Knockdown of TLR4 expression resulted in a significant decrease in sCD14 transfer across Caco-2 monolayers. Specifically, in control Caco-2 cells, $3.45 \pm 0.13\%$ of hrCD14 was transferred to the basal media, whereas $0.005 \pm 0.001\%$ of hrCD14 was transferred from the apical to basal media in TLR-siRNA treated cells ($P < 0.001$, Fig. 7B).

Additionally, the role of TLR4 in CD14 transfer was monitored by microscopy (Fig. 8). Regardless of siRNA treatment, Caco-2

cells were able to internalize hrCD14 upon exposure (no hrCD14 compared to hrCD14 exposure, $P < 0.037$, Fig. 8). Interestingly, the amount of sCD14 internalized did not significantly change despite knockdown of TLR4 ($P > 0.135$, Fig. 4C). Therefore, knockdown of TLR4 seems to impair transfer of CD14 across monolayers (Fig. 7), but not uptake of CD14 (Fig. 8).

Discussion

sCD14 from milk is transferred to the blood of pups

Ingested sCD14 was transferred to the blood of mice as shown by detection of CD14 in the blood of CD14^{-/-} pups fostered to WT mothers ($0.16 \pm 0.09 \mu\text{g/mL}$, day eight PP, Fig. 1F). Ingested sCD14 contributed approximately 26.7% of the circulating sCD14 on day eight PP, as assessed by comparing the concentration of sCD14 in the blood of WT pups fostered to WT mothers to CD14^{-/-} pups fostered to WT mothers ($0.60 \pm 0.14 \mu\text{g/mL}$ and $0.16 \pm 0.09 \mu\text{g/mL}$,

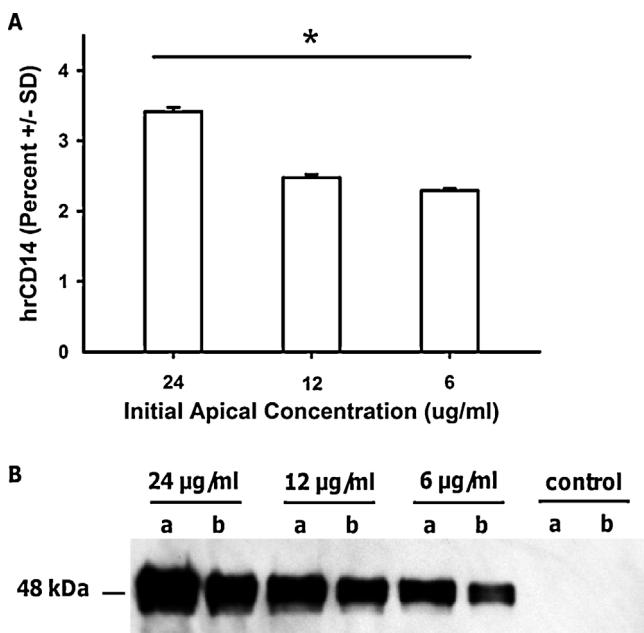


Fig. 4. Quantity (A) and intactness (B) of sCD14 transferred across human intestinal monolayers *in vitro*. Caco-2 cells were grown and differentiated as monolayers in Transwell assays. Human recombinant CD14 (hrCD14) was added to apical medium and transfer of hrCD14 to basal medium was measured by ELISA. Intactness of hrCD14 in the apical (a) and basal (b) medium was detected by western blot. *, $P < 0.05$ by *t*-test, $n = 3$.

respectively, Fig. 1). The contribution of ingested sCD14 to circulating levels of sCD14 pups was significant earlier in lactation, when sCD14 levels in milk were higher (Fig. 1A). Specifically, when comparing WT pups fostered to CD14 $^{-/-}$ mothers to WT pups fostered to WT mothers, the amount of circulating sCD14 significantly decreased from $0.60 \pm 0.14 \mu\text{g}/\text{mL}$ at day eight PP (WT to WT) to $0.35 \pm 0.05 \mu\text{g}/\text{mL}$ at day eight PP (WT to CD14 $^{-/-}$) due to the absence of sCD14 from their diet ($P = 0.016$, Fig. 1B and D). Over time, the amount of sCD14 in the mouse milk decreased to non-detectable levels, but the level of circulating sCD14 in WT pups did not show a similar decrease (Fig. 1). For example, when comparing sCD14 levels in the blood of WT pups fostered to WT mothers to WT pups fostered to CD14 $^{-/-}$ mothers, the amount of circulating sCD14 on days nine and ten PP do not significantly differ between the groups regardless of the absence of sCD14 from the diet of the WT pups fostered to CD14 $^{-/-}$ mothers ($P > 0.05$, Fig. 1B and D). The lack of correlation between milk-sCD14 levels and circulating sCD14 levels after eight days of suckling suggests pups begin producing more of their own circulating sCD14 at this time and rely less on contributions from ingested sCD14.

A decrease in sCD14 levels during lactation is also seen in humans, where sCD14 levels are reported as high as $26 \mu\text{g}/\text{mL}$ in colostrum, which decreases to $5 \mu\text{g}/\text{mL}$ by 30 days PP (Collado et al., 2012). Additionally, circulating sCD14 levels in infants do not reach levels similar to those in adults until four months PP (Jones et al., 2002). Therefore, uptake of ingested sCD14 by infants as a means to compensate for a lack of endogenously produced sCD14 is likely a conserved mechanism in both rodents and humans. This is supported by the observation of human intestinal cells transferring hrCD14 across monolayers *in vitro* (Fig. 4). When exposed to hrCD14, Caco-2 cells were able to internalize and transfer hrCD14 from the apical to basal well of a Transwell assay in a dose-dependent manner (Fig. 4).

Caco-2 monolayers have also been used as a model to demonstrate the transcytosis of other milk components including proteins, bacteria and viruses. For example β -lactoglobulin, a

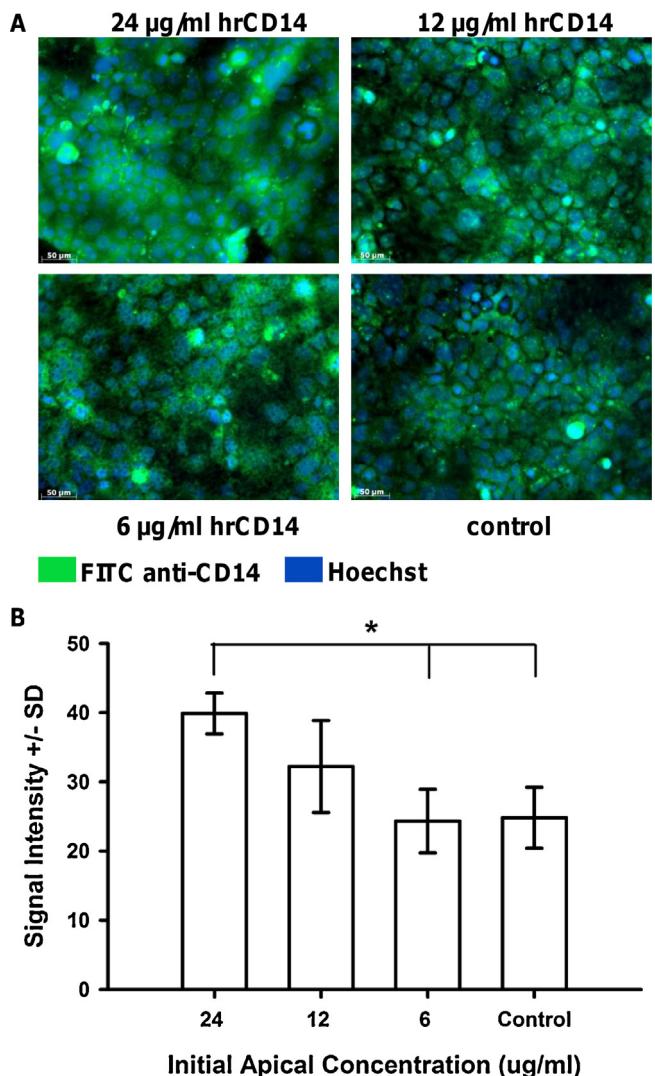


Fig. 5. Visualization of sCD14 within human intestinal cells treated with sCD14 *in vitro*. Caco-2 cells were grown and differentiated as monolayers in Transwell assays. Human recombinant CD14 (hrCD14) was added to the apical medium and presence of hrCD14 within the monolayers was detected by microscopy (A) after 2 h of incubation with or without hrCD14. Signal intensities for hrCD14 (B) were calculated using ImageJ. *, $P < 0.05$ by *t*-test, $n = 3$.

major bovine milk allergen, was recently shown to evade digestion similarly to sCD14, and was also transferred across Caco-2 monolayers *in vitro* (Picariello et al., 2013). Viruses found in human milk, such as human T-cell leukemia virus type 1 (HTLV-1) which resides within infected milk-lymphocytes in HTLV-1 positive mothers, has also been shown to transfer across Caco-2 monolayers. This transfer occurs via transcytosis despite the resistance of enterocytes to HTLV-1 infection (Martin-Latil et al., 2012). Similarly, Caco-2 monolayers and brain microvascular endothelial cell monolayers have been shown to transcytose *Cronobacter*, a type of bacteria linked to neonatal meningitis (Giri et al., 2012). *Cronobacter* can be found in human milk (Ward et al., 2013) and can also reside within powdered infant formulas, the latter of which has been reported as a source of neonatal meningitis outbreaks and discussed in a recent review (Norberg et al., 2012).

In vivo uptake of other milk proteins, such as immunoglobulins, has also been extensively studied. Recently, transcytosis of IgG by intestinal cells of rats was imaged by electron tomography, which showed the antibody to be taken up by epithelial cells along the intestine and transported to the blood in an endosome dependent

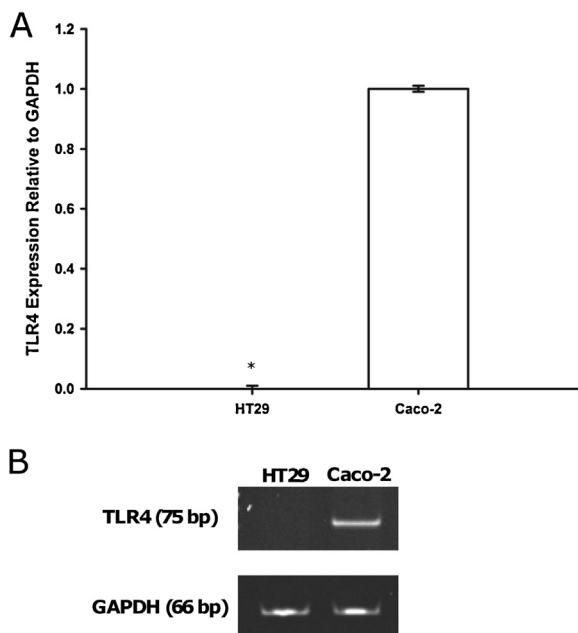


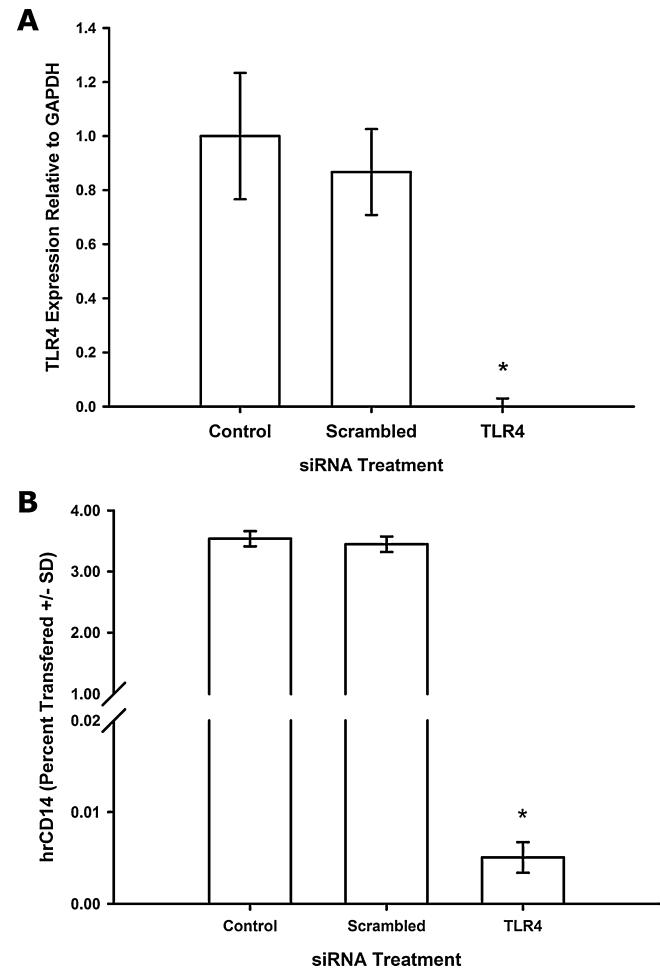
Fig. 6. TLR4 expression in Caco-2 and HT29 cells. Intestinal epithelial cell lines were grown in monolayers and TLR4 expression was determined by real time PCR (A). TLR4 (75 bp) and GAPDH (66 bp) amplicons were electrophoresed following PCR on polyacrylamide gels (B).

pathway (He et al., 2008; Ladinsky et al., 2012). Further analysis revealed the intestinal cells of weaned animals had less multivesicular bodies containing early endosomal markers in comparison to neonatal animals (Ladinsky et al., 2012). The change in vesicle phenotype throughout suckling suggests the capacity of the neonatal intestine to transport functional proteins from the lumen of the intestine and into the blood diminishes during maturation. The present study observed sCD14 transport across a window of five days (days six through ten PP) and thus changes in sCD14 transport across the GI tract over time were likely not impacted greatly by gut maturation (Fig. 1).

sCD14 transferred to the blood remains functional

One clearly demonstrated function of CD14 (both mCD14 and sCD14) is its ability to recognize LPS from Gram-negative bacteria and initiate an innate immune response through TLR4 (Wright et al., 1990). Because of this, CD14^{-/-} mice are resistant to LPS mediated septic shock, as minimal TNF- α and IL-6 responses are observed following injection of LPS in concentrations normally lethal to WT mice (Haziot et al., 1996). When CD14^{-/-} pups were fostered to WT mothers, their level of circulating sCD14 increased as ingested milk-sCD14 was transferred to the blood (Fig. 1F). When injected with LPS, CD14^{-/-} pups fostered to WT mothers presented with significantly higher TNF- α and IL-6 responses than CD14^{-/-} pups fostered CD14^{-/-} mothers ($P < 0.05$, Fig. 3B and C). This suggests that ingested sCD14 transferred to the blood of pups retains its ability to detect LPS.

CD14^{-/-} pups fostered to WT mothers had a significantly lower immune response (both IL-6 and TNF- α) following LPS injection in comparison to WT pups fostered to WT mothers ($P = 0.002$, Fig. 3B and $P = 0.007$, Fig. 3C). This is likely due to the presence of only sCD14 and no mCD14 in the CD14^{-/-} pups fostered to WT mothers. In WT mice, mCD14 can be found on monocytes, hepatocytes and intestinal epithelial cells where it is responsible for recognizing LPS and initiating the TLR4 mediated immune response (Guo et al., 2013; Wang et al., 1998). Therefore, because mCD14 is found



on numerous cells in WT pups, WT pups are more readily equipped to recognize LPS regardless of sCD14 ingestion. When comparing the immune response to LPS of WT pups fostered to WT mothers (WT to WT) to WT pups fostered to CD14^{-/-} mothers (WT to CD14^{-/-}), there was a significantly higher IL-6 response in WT pups fostered to CD14^{-/-} mothers ($P = 0.030$, Fig. 3C). On the second day post-foster, significantly less sCD14 was observed in the blood of WT pups fostered to CD14^{-/-} mothers in comparison to WT pups fostered to WT mothers ($P = 0.016$, Fig. 1B and D). As previously stated, WT pups express mCD14 on various cell types which are then able to detect LPS, regardless of sCD14 ingestion. Although milk sCD14 is capable of recognizing LPS and subsequently passing LPS to TLR4 (Vidal et al., 2001), sCD14 can also sequester LPS away from mCD14 expressing cells and bring LPS to the liver for clearance by TLR4 expressing hepatocytes (Deng et al., 2013; Kitchens et al., 2001). Therefore, the lower levels of sCD14 in WT pups fostered to CD14^{-/-} mothers may decrease the amount of LPS sent to the liver for clearance, resulting in a higher immune response to LPS exposure.

sCD14 transfer by Caco-2 cells involves TLR4

Caco-2 cells in Transwell assays were able to transfer hrCD14 across the cell monolayer from the apical medium to the basal

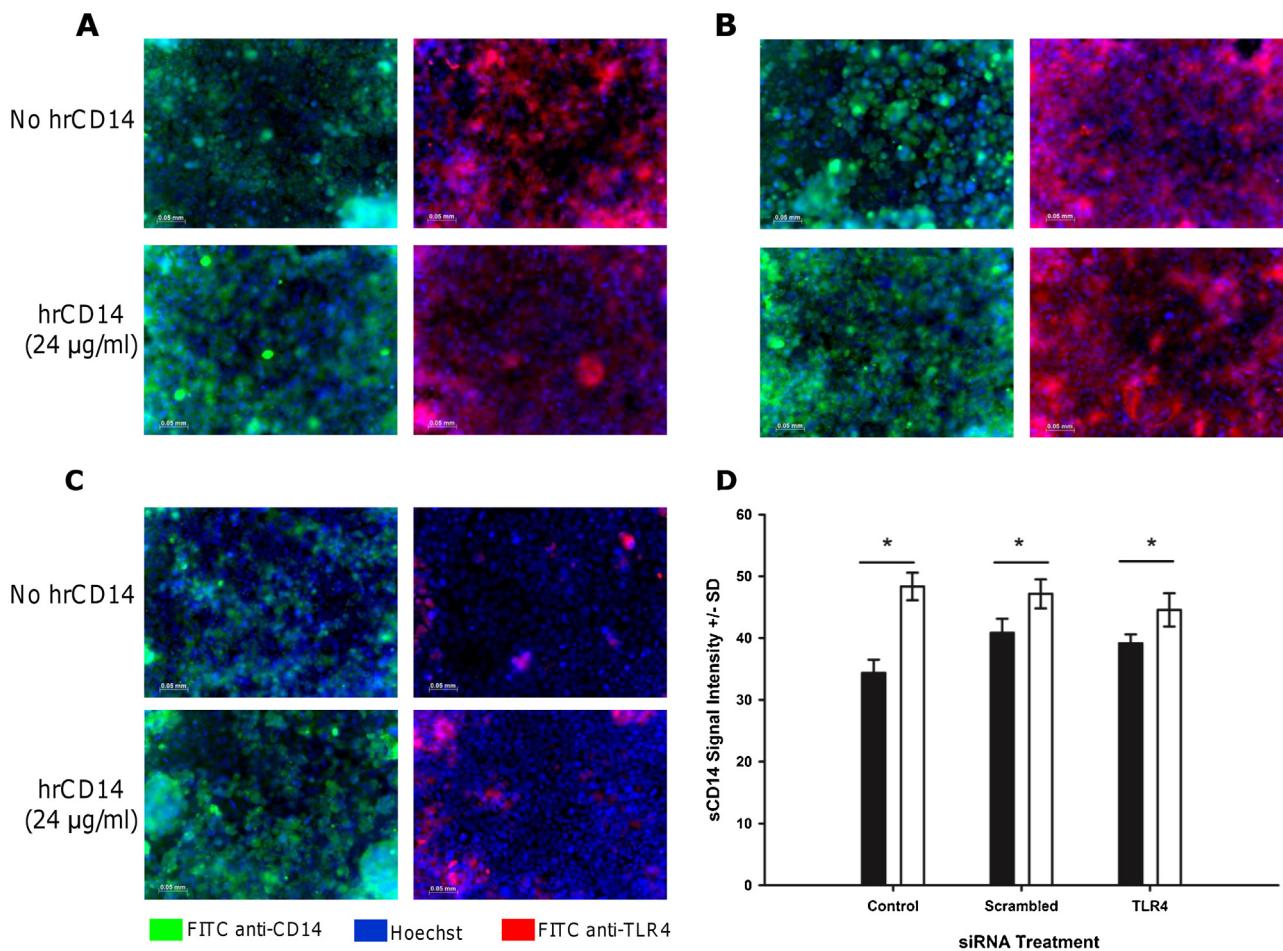


Fig. 8. Visualization of sCD14 and TLR4 within human intestinal cells treated with siRNA. Caco-2 cells were grown and differentiated as monolayers in Transwell assays and treated without siRNA (A), scrambled siRNA (B), or TLR4 specific siRNA (C). Uptake of human recombinant CD14 (hrCD14, left panels) and TLR4 expression (right panels) were detected by microscopy. Signal intensities for hrCD14 (D) were calculated using ImageJ. Black bars represent cells given no hrCD14, white bars represent cells treated with hrCD14.*, $P < 0.05$ by t-test, $n = 3$.

medium in a dose dependent manner (Fig. 4). Previous research has shown CD14 internalization to be independent of TLR4 (Zanoni et al., 2011), however, given the role of CD14 in LPS recognition and TLR4 signaling, we hypothesized CD14 internalization by intestinal cells may involve TLR4. When TLR4 expression was knocked down using siRNA, the capacity of Caco-2 cells to transport hrCD14 across the monolayer was diminished ($3.45 \pm 0.13\%$ of hrCD14 transferred by control cells versus $0.005 \pm 0.001\%$ of hrCD14 transferred by TLR4-siRNA treated cells, $P < 0.001$, Fig. 7B). Interestingly, although the amount of hrCD14 transferred across the monolayers was significantly less following TLR4 knockdown, the amount of hrCD14 internalized (as measured by microscopy) did not significantly change ($P > 0.135$, Fig. 8). Therefore, TLR4 may be involved in the transfer of TLR4 to the basal side of cell monolayers as opposed to apical uptake of CD14. The exact role of TLR4 in the transfer of CD14 across intestinal cells and whether TLR4 plays a role in sCD14 transfer *in vivo*, however, remains unclear.

In rats, LPS transport across the intestinal epithelium *ex vivo* was observed to be inhibited using anti-CD14 or anti-TLR4 antibodies, supporting a role of these two pattern recognition receptors in mucosal LPS transport (Tomita et al., 2004). Similarly, a study using mouse intestinal cells *in vitro* (m-IC_{c12}) showed an increase of CD14 expression following LPS exposure, and LPS was internalized and colocalized with TLR4 within m-IC_{c12} cells (Hornef et al., 2002). Recently, a study with Caco-2 cells showed that LPS increased TLR4 and CD14 expression in intestinal monolayers, and a similar trend

was observed *in vivo* along the mouse GI tract (Guo et al., 2013). In healthy intestine, LPS does not permeate through the intestinal barrier to a great extent (intestinal lumen to blood transfer), likely due to a low presence of TLR4 and CD14 along the GI tract (Guo et al., 2013; Leaphart et al., 2007; Nanthakumar et al., 2011; Wolfs et al., 2010). Once exposed to LPS, TLR4 and CD14 concentrations increase, resulting in greater intestinal permeability (Guo et al., 2013). It is likely that milk-derived sCD14 is able to cross the intestinal barrier through involvement with LPS recognition, given the demonstrated role of TLR4 in CD14 transfer *in vitro* (Fig. 7).

Implications of biologically active sCD14 post-ingestion

Although high concentrations of sCD14 in early-lactation milk in conjunction with sCD14's ability to transfer to the blood post-ingestion suggest sCD14 is an important human milk protein, sCD14's role in infant health remains unknown. Similarly to following LPS exposure as shown by Guo et al, TLR4 is also upregulated along the GI tract of premature infants (Guo et al., 2013; Leaphart et al., 2007; Nanthakumar et al., 2011; Wolfs et al., 2010). Once activated, TLR4 is able to induce a proinflammatory response, and this activation in the intestine has been implicated as a cause of NEC (Hackam et al., 2013; Neu and Walker, 2011 for review). Incidence of NEC is higher in premature infants and lower birth weight infants have the highest NEC-caused death rates of up to 30% (Fitzgibbons et al., 2009). During NEC, activation of TLR4 leads to autophagy

and subsequent impaired migration of intestinal epithelial cells from the crypts of the intestine resulting in decreased barrier integrity (Neal et al., 2013). Additionally, TLR4 activation within the endothelium has recently been shown to reduce mesenteric perfusion resulting in intestinal ischemia and increased NEC severity (Yazji et al., 2013). Activation of TLR4 may be dependent on CD14, since blocking CD14 through inactivating antibodies decreased the severity of induced NEC by decreasing the TLR4 immune response as measured by IL-6 and TNF- α expression (Chan et al., 2009). WT pups not ingesting sCD14 were seen to have an increase in IL-6 response to LPS compared to those ingesting sCD14 (Fig. 3), which confirms previous studies that show increased sCD14 levels prior to or at the time of LPS exposure dampen the LPS-induced immune response (Haziot et al., 1995; Lee et al., 2003). Therefore, high levels of sCD14, such as those in colostrum and early lactation milk (26 μ g/mL), may be beneficial to the infant prior to exposure of LPS to help mitigate an exaggerated immune response.

Conclusions

Ingestion of sCD14 within milk results in the transfer of intact sCD14 from the GI tract to the circulatory system of mouse pups. Transfer of sCD14 across the intestinal barrier involves TLR4 *in vitro* and newly circulating sCD14 derived from ingested sources remains functional in its ability to detect LPS *in vivo*. High quantities of sCD14 in early lactation milk implicate sCD14 as a factor influencing infant health, especially given the role of the LPS recognition pathway, which includes CD14 and TLR4, in the development of NEC.

Conflict of interest

The authors declare no conflict of interest.

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References

- Blais, D.R., Vascotto, S.C., Griffith, M., Altosaar, I., 2005. LBP and CD14 secreted in tears by the lacrimal glands modulate the LPS response of corneal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **46**, 4235–4244.
- Chan, K.L., Wong, K.F., Luk, J.M., 2009. Role of LPS/CD14/TLR4-mediated inflammation in necrotizing enterocolitis: pathogenesis and therapeutic implications. *World J. Gastroenterol.* **15**, 4745–4752.
- Collado, M.C., Laitinen, K., Salminen, S., Isolauri, E., 2012. Maternal weight and excessive weight gain during pregnancy modify the immunomodulatory potential of breast milk. *Pediatr. Res.* **72**, 77–85.
- Deng, M., Scott, M.J., Loughran, P., Gibson, G., Sodhi, C., Watkins, S., Hackam, D., Billiar, T.R., 2013. Lipopolysaccharide clearance, bacterial clearance, and systemic inflammatory responses are regulated by cell type-specific functions of TLR4 during sepsis. *J. Immunol.* **190**, 5152–5160.
- Fitzgibbons, S.C., Ching, Y., Yu, D., Carpenter, J., Kenny, M., Weldon, C., Lillehei, C., Valim, C., Horbar, J.D., Jaksic, T., 2009. Mortality of necrotizing enterocolitis expressed by birth weight categories. *J. Pediatr. Surg.* **44**, 1072–1075.
- Giri, C.P., Shima, K., Tall, B.D., Curtis, S., Sathyamoorthy, V., Hanisch, B., Kim, K.S., Kopecko, D.J., 2012. *Cronobacter* spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells. *Microb. Pathog.* **52**, 140–147.
- Guo, S., Al-Sadi, R., Said, H.M., Ma, T.Y., 2013. Lipopolysaccharide causes an increase in intestinal tight junction permeability *in vitro* and *in vivo* by inducing enteroocyte membrane expression and localization of TLR-4 and CD14. *Am. J. Pathol.* **182**, 375–387.
- Hackam, D.J., Afrazi, A., Good, M., Sodhi, C.P., 2013. Innate immune signaling in the pathogenesis of necrotizing enterocolitis. *Clin. Dev. Immunol.* **2013**, 475415.
- Haziot, A., Rong, G.W., Lin, X.Y., Silver, J., Goyert, S.M., 1995. Recombinant soluble CD14 prevents mortality in mice treated with endotoxin (lipopolysaccharide). *J. Immunol.* **154**, 6529–6532.
- Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L., Goyert, S.M., 1996. Resistance to endotoxin shock and reduced dissemination of Gram-negative bacteria in CD14-deficient mice. *Immunity* **4**, 407–414.
- Haziot, A., Hijiya, N., Gangloff, S.C., Silver, J., Goyert, S.M., 2001. Induction of a novel mechanism of accelerated bacterial clearance by lipopolysaccharide in CD14-deficient and Toll-like receptor 4-deficient mice. *J. Immunol.* **166**, 1075–1078.
- He, W., Ladinsky, M.S., Huey-Tubman, K.E., Jensen, G.J., McIntosh, J.R., Bjorkman, P.J., 2008. FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. *Nature* **455**, 542–546.
- Hornef, M.W., Frisan, T., Vandewalle, A., Normark, S., Richter-Dahlfors, A., 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* **195**, 559–570.
- Jones, C.A., Holloway, J.A., Popplewell, E.J., Diaper, N.D., Holloway, J.W., Vance, G.H.S., Warner, J.A., Warner, J.O., 2002. Reduced soluble CD14 levels in amniotic fluid and breast milk are associated with the subsequent development of atopy, eczema, or both. *J. Allergy Clin. Immunol.* **109**, 858–866.
- Kagan, J.C., Medzhitov, R., 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* **125**, 943–955.
- Kagan, J.C., Su, T., Horng, T., Chow, A., Akira, S., Medzhitov, R., 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat. Immunol.* **9**, 361–368.
- Kitchens, R.L., Thompson, P.A., Viriyakosol, S., O'Keefe, G.E., Munford, R.S., 2001. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. *J. Clin. Invest.* **108**, 485–493.
- Ladinsky, M.S., Huey-Tubman, K.E., Bjorkman, P.J., 2012. Electron tomography of late stages of FcRn-mediated antibody transcytosis in neonatal rat small intestine. *Mol. Biol. Cell.* **23**, 2537–2545.
- Leaphart, C.L., Cavallo, J., Gribar, S.C., Cetin, S., Li, J., Branca, M.F., Dubowski, T.D., Sodhi, C.P., Hackam, D.J., 2007. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J. Immunol.* **179**, 4808–4820.
- Lee, J.W., Paape, M.J., Zhao, X., 2003. Recombinant bovine soluble CD14 reduces severity of experimental *Escherichia coli* mastitis in mice. *Vet. Res.* **34**, 307–316.
- Maayan-Metzger, A., Itzhak, A., Mazkereth, R., Kuint, J., 2004. Necrotizing enterocolitis in full-term infants: case-control study and review of the literature. *J. Perinatol.* **24**, 494–499.
- Martin-Latil, S., Gnagid, N.F., Mallet, A., Desdouits, M., Guivel-Benhassine, F., Jeannin, P., Prevost, M.C., Schwartz, O., Gessain, A., Ozden, S., Ceccaldi, P.E., 2012. Transcytosis of HTLV-1 across a tight human epithelial barrier and infection of subepithelial dendritic cells. *Blood* **120**, 572–580.
- Nanthakumar, N., Meng, D., Goldstein, A.M., Zhu, W., Lu, L., Uauy, R., Llanos, A., Claud, E.C., Walker, W.A., 2011. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS ONE* **6**, e17776.
- Neal, M.D., Sodhi, C.P., Dyer, M., Craig, B.T., Good, M., Jia, H., Yazji, I., Afrazi, A., Richardson, W.M., Beer-Stoltz, D., Ma, C., Prindle, T., Grant, Z., Branca, M.F., Ozolek, J., Hackam, D.J., 2013. A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. *J. Immunol.* **190**, 3541–3551.
- Neu, J., Walker, W.A., 2011. Necrotizing enterocolitis. *N. Engl. J. Med.* **364**, 255–264.
- Norberg, S., Stanton, C., Ross, R.P., Hill, C., Fitzgerald, G.F., Cotter, P.D., 2012. *Cronobacter* spp. in powdered infant formula. *J. Food Prot.* **75**, 607–620.
- Picariello, G., Iacominio, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., Di Luccia, A., Addeo, F., 2013. Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins. *Food Chem.* **139**, 203–212.
- Sanchez-Munoz, F., Fonseca-Camarillo, G., Villeda-Ramirez, M.A., Miranda-Perez, E., Mendivil, E.J., Barreto-Zuniga, R., Uribe, M., Bojalil, R., Dominguez-Lopez, A., Yamamoto-Furusho, J.K., 2011. Transcript levels of Toll-like receptors 5, 8 and 9 correlate with inflammatory activity in ulcerative colitis. *BMC Gastroenterol.* **11**, 138.
- Sullivan, S., Schanler, R.J., Kim, J.H., Patel, A.L., Trawoger, R., Kiechl-Kohlendorfer, U., Chan, G.M., Blanco, C.L., Abrams, S., Cotten, C.M., Laroia, N., Ehrenkranz, R.A., Dudell, G., Cristofalo, E.A., Meier, P., Lee, M.L., Rechtman, D.J., Lucas, A., 2010. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *J. Pediatr.* **156**, 562–567.
- Tomita, A., Ohkubo, R., Hayashi, M., 2004. Lipopolysaccharide transport system across colonic epithelial cells in normal and infective rat. *Drug Metab. Pharmacokinet.* **19**, 33–40.
- Vamadevan, A.S., Fukata, M., Arnold, E.T., Thomas, L.S., Hsu, D., Abreu, M.T., 2010. Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. *Innate Immun.* **16**, 93–103.
- Vidal, K., Labeta, M.O., Schiffrian, E.J., Donnet-Hughes, A., 2001. Soluble CD14 in human breast milk and its role in innate immune responses. *Acta Odontol. Scand.* **59**, 330–334.

- Wang, S.C., Klein, R.D., Wahl, W.L., Alarcon, W.H., Garg, R.J., Remick, D.G., Su, G.L., 1998. Tissue coexpression of LBP and CD14 mRNA in a mouse model of sepsis. *J. Surg. Res.* 76, 67–73.
- Ward, T.L., Hosid, S., Ioshikhes, I., Altosaar, I., 2013. Human milk metagenome: a functional capacity analysis. *BMC Microbiol.* 13, 116.
- Ward, T.L., Spencer, W.J., Davis, L.D., Harrold, J., Mack, D.R., Altosaar, I., 2014. Ingested soluble CD14 from milk is transferred intact into the blood of newborn rats. *Pediatr. Res.* 75, 252–258.
- Wolfs, T.G., Derikx, J.P., Hodin, C.M., Vanderlocht, J., Driessens, A., de Bruine, A.P., Bevins, C.L., Lasitschka, F., Gassler, N., van Gemert, W.G., Buurman, W.A., 2010. Localization of the lipopolysaccharide recognition complex in the human healthy and inflamed premature and adult gut. *Inflamm. Bowel Dis.* 16, 68–75.
- Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249, 1431–1433.
- Yazji, I., Sodhi, C.P., Lee, E.K., Good, M., Egan, C.E., Afrazi, A., Neal, M.D., Jia, H., Lin, J., Ma, C., Branca, M.F., Prindle, T., Richardson, W.M., Ozolek, J., Billiar, T.R., Binion, D.G., Gladwin, M.T., Hackam, D.J., 2013. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signaling. *Proc. Natl. Acad. Sci. U. S. A.* 110, 9451–9456.
- Zanoni, I., Ostuni, R., Marek, L.R., Barresi, S., Barbalat, R., Barton, G.M., Granucci, F., Kagan, J.C., 2011. CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147, 868–880.