Dietary Depletion of Milk Exosomes and Their MicroRNA Cargos Elicits a Depletion of miR-200a-3p and Elevated Intestinal Inflammation and Chemokine (C-X-C Motif) Ligand 9 Expression in *Mdr1a*$^{-/-}$ Mice

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ABSTRACT

Background: Exosomes transfer regulatory microRNAs (miRs) from donor cells to recipient cells. Exosomes and miRs originate from both endogenous synthesis and dietary sources such as milk. miR-200a-3p is a negative regulator of the proinflammatory chemokine (C-X-C motif) ligand 9 (CXCL9). Male *Mdr1a*$^{-/-}$ mice spontaneously develop clinical signs of inflammatory bowel disease (IBD).

Objectives: We assessed whether dietary depletion of exosomes and miRs alters the severity of IBD in *Mdr1a*$^{-/-}$ mice owing to aberrant regulation of proinflammatory cytokines.

Methods: Starting at 5 wk of age, 16 male *Mdr1a*$^{-/-}$ mice were fed either milk exosome– and RNA-sufficient (ERS) or milk exosome– and RNA-depleted (ERD) diets. The ERD diet is characterized by a near-complete depletion of miRs and a 60% loss of exosome bioavailability compared with ERS. Mice were killed when their weight loss exceeded 15% of peak body weight. Severity of IBD was assessed by histopathological evaluation of cecum. Serum cytokine and chemokine concentrations and mRNA and miR tissue expression were analyzed by multiplex ELISAs, RNA-sequencing analysis, and qRT-PCR, respectively.

Results: Stromal collapse, gland hyperplasia, and additive microscopic disease scores were (mean ± SD) 56.7% ± 23.3%, 23.5% ± 11.8%, and 29.6% ± 8.2% lower, respectively, in ceca of ERS mice than of ERD mice ($P < 0.05$). The serum concentration of CXCL9 was 35.0% ± 31.0% lower in ERS mice than in ERD mice ($P < 0.05$). Eighty-seven mRNAs were differentially expressed in the ceca from ERS and ERD mice; 16 of these mRNAs are implicated in immune function. The concentrations of 4 and 1 out of 5 miRs assessed (including miR-200a-3p) were ≤63% lower in livers and ceca, respectively, from ERD mice than from ERS mice.

Conclusions: Milk exosome and miR depletion exacerbates cecal inflammation in *Mdr1a*$^{-/-}$ mice.   *Curr Dev Nutr* 2019;3:nzz122.

Keywords: CXCL9, exosomes, inflammatory bowel disease, *Mdr1a*$^{-/-}$ mice, microRNA

Introduction

Most living cells secrete exosomes into the extracellular space (1, 2). Exosomes are present in body fluids such as milk, urine, serum, and plasma (3). Currently, the exosome community distinguishes ≥3 distinct populations of exosomes, i.e., small exosomes (60–80 nm diameter), large exosomes (90–120 nm), and exomeres (a class of nonmembranous exosomes, ~35 nm) (1, 2, 4). Exosomes participate in cell-to-cell communication, which is facilitated by the transfer of regulatory exosome cargos from donor cells to recipient cells (1, 2). Among regulatory exosome cargos, microRNA (miR) cargos have attracted particular attention, because miRs regulate the expression of >60% of human genes (5).

Evidence suggests that exosomes and their miR cargos do not exclusively originate from endogenous synthesis but may also be obtained from dietary sources such as milk. For example, we have reported that

consumption of bovine milk causes a postprandial increase in plasma miR concentrations in humans, including bovine miRs that humans cannot synthesize (6–8). We have further reported that bovine milk exosomes and their cargos are transported across the intestinal mucosa and taken up by vascular endothelial cells in human and rodent cell cultures (9, 10). When bovine milk exosomes were labeled with fluorescent dyes, fluorescent fusion proteins, or fluorophore-labeled miRs and administered orally to mice, the exosomes and their miR cargos accumulated in liver, brain, and, to a lesser extent, in peripheral tissues such as spleen, kidneys, lung, and heart (11). Although some groups disputed the bioavailability of milk miRs (but not that of exosomes; see Discussion), the bioavailability of miRs in milk has been confirmed by ≥5 independent laboratories (6, 11–17). Importantly, when mice were fed an exosome- and RNA-depleted diet (ERD), plasma miR concentrations decreased by >60% compared with controls fed an exosome- and RNA-sufficient (ERS) diet, suggesting that endogenous synthesis cannot compensate for dietary depletion [(6), see Methods]. Consumption of ERD elicited also phenotypes such as aberrant purine metabolism and moderate loss of muscle grip strength compared with ERS controls in human infants and adults and mice in previous studies (18, 19).

Bovine milk exosomes contain immune-related miRs in quantities sufficient to alter human immune function, including miR-200a-3p (20–22). Some of these miRs are implicated in gut health. For example, miR-146b alleviates intestinal inflammation by activating NF-κB in an IL-10-deficient mouse colitis model, and miR-200b promotes proliferation of intestinal epithelial cells through inhibiting the epithelial–mesenchymal transition via transforming growth factor-β (23, 24). In the context of this project, miR-200a-3p is of particular interest. The 3'-untranslated region (3'-UTR) of the chemokine (C-X-C motif) ligand 9 (CXCL9) harbors a putative miR-200a-3p binding site and participates in the recruitment of leukocytes to sites of inflammation in the gut (25).

Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn disease are chronic inflammatory disorders of the digestive tract caused and exacerbated by genetic mutations, aberrant immune responses to gut microbial antigens, and environmental factors (26). *Mdr1a* knockout (*Mdr1a*$^{-/-}$) mice lack the murine multiple drug resistance gene coding for P-glycoprotein 170, and male *Mdr1a*$^{-/-}$ mice spontaneously develop severe signs of IBD by ∼12 wk of age (27). The IBD phenotype is similar in *Mdr1a*$^{-/-}$ mice and humans, and *Mdr1a*$^{-/-}$ mice are an established animal model in studies of IBD.

Here, we tested the hypothesis that dietary depletion of exosomes and their miR cargos alters the severity of IBD in *Mdr1a*$^{-/-}$ mice. The objectives of our study were to assess the effects of milk exosomes and miRs on the severity of IBD and to assess the molecular mechanisms that mediate any such effect.

Methods

Mice

Four-week-old *Mdr1a*$^{-/-}$ mice and *Mdr1a*$^{+/+}$ controls were obtained from Taconic Biosciences, Inc. and acclimated for 1 wk before study initiation. *Mdr1a*$^{-/-}$ mice are in the FVB genetic background, and FVB wild-type mice are denoted as *Mdr1a*$^{+/+}$ mice in this article. Male mice were selected for study because clinical signs of IBD are absent in *Mdr1a*$^{-/-}$ females, or less severe than in males (26, 28).

Experimental diets

ERS and ERD diets were used for this study (6, 18). Diets were based on the AIN-93G formula and modified by their content of exosomes and their RNA cargos from bovine milk (6, 29). In the diets, soy protein and skim milk (ERS) and ultrasonicated skim milk (ERD) substituted for milk casein in the AIN-93G formulation to control the consumption of dairy exosomes and their RNA cargos. Ultrasonication of milk leads to a >98% depletion of miRs in milk exosomes, a 20% depletion of exosomes in milk, and a >60% decrease in exosome uptake by Caco-2 cells compared with exosomes from nonsonicated milk (18, 30). The observed loss of miRs is consistent with a previous study suggesting that opening up exosome membranes causes a rapid degradation of miRs (31). The milk added to the diets provided the equivalent of 0.5 L milk consumed by a human adult per day, adjusted by body weight in mice. Diet ingredients other than milk were not ultrasonicated, i.e., nutrients other than exosomes and their RNA cargos were the same in both ERD and ERS diets. Diet ingredients were pelleted before feeding.

Experimental design

At 5 wk of age, *Mdr1a*$^{-/-}$ and *Mdr1a*$^{+/+}$ mice were randomly assigned to ERS or ERD diets (n = 8/group). One mouse in group ERS *Mdr1a*$^{-/-}$ died of causes apparently not related to IBD (n = 7) and was excluded from analysis. Body weight was monitored at timed intervals and *Mdr1a*$^{-/-}$ mice were killed for sample collection when weight loss exceeded 15% of peak body weight owing to animal welfare concerns; *Mdr1a*$^{+/+}$ controls were removed at the same time when the last *Mdr1a*$^{-/-}$ mice were removed from study. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Nebraska–Lincoln (protocol 1215).

Sample collection

Thirty-one mice were killed by carbon dioxide inhalation. Blood was harvested via cardiac puncture, and serum was obtained by centrifugation (9500 × g for 5 min at room temperature) of blood allowed to clot at room temperature for 30 min. Cecum and colon were excised and washed with cold PBS, and colon length was measured using a ruler. The top 1.5 cm of cecum including the cecal tonsil and the most distal 1 cm of colon were stored in 10% (3.63 mol/L) formaldehyde for histopathology analysis. The remainder of the cecum was snap-frozen in liquid nitrogen for RNA-sequencing analysis.

Histopathological analysis

Formaldehyde-fixed cecum and colon samples were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined via light microscopy by a board-certified veterinary pathologist in the Comparative Pathology Core facility at Iowa State University. The pathologist was blinded to experimental treatment and assigned scores to tissues based on mucosal height, ulceration, inflammation score, edema, stromal collapse, and gland hyperplasia as previously described (32). Values for each parameter were added together to generate a cumulative histopathological score, with larger values representing more severe disease. Tissue slides that failed a quality assessment by the pathologist were excluded from scoring; 5–8 tissues were scored per genotype and dietary treatment.

Cytokine and chemokine analysis

Concentrations of cytokines and chemokines in serum were measured using the Millipore Mouse Cytokine/chemokine Magnetic Bead Multiplex Assay Kit and a MAGPIX instrument (Luminex Corporation). The assay captures eotaxin, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (subunit 40 kDa), IL-12 (subunit 70 kDa), IL-13, IL-15, IL-17, IFN-γ-induced protein 10, keratinocyte chemoattractant, leukemia inhibitory factor, lipopolysaccharide-induced CXC chemokine, monocyte chemoattractant protein 1, macrophage colony stimulating factor, CXCL9, macrophage inflammatory protein 1α, macrophage inflammatory protein 1β, macrophage inflammatory protein 2, chemokine (C-C motif) ligand 5, TNF-α, and vascular endothelial growth factor.

RNA-sequencing analysis

Cecal mRNA was isolated using the miRNeasy mini kit (Qiagen Inc.). Libraries were created using the TrueSeq RNA Sample Preparation V2 Kit (Illumina, Inc.) and RNA-sequencing analysis was conducted in the University of Nebraska Medical Center Sequencing Core using the NextSeq 500 sequencer platform and a 150-bp single-end protocol (18). Sequencing data were analyzed as previously described (18). EBseq was used to identify transcripts that were differentially expressed in *Mdr1a*$^{-/-}$ ERS and ERD mice (33). Gene expression is presented in units of normalized expected counts, which indicates reads per kilobase of transcript per million mapped reads of an mRNA. Data were deposited in the BioProject database under accession ID PRJNA516639.

Analysis of miRs by qRT-PCR

Putative miR binding sites in the 3′-UTR of Cxcl9 mRNA were predicted by using Target Scan Mouse 7.1 (34). Cxcl9 mRNA contains 503 putative miR binding sites including 1 binding site for miR-200a-3p (**Supplemental Figure 1**); complementarity between miR-200a-3p and the 3′-UTR in murine Cxcl9 mRNA is 100% for the seed region but only 59% for the entire miR-200a-3p. We assessed the expression of 5 miRs including miR-200a-3p to assess whether dietary miR depletion causes a depletion of miRs in ceca and livers (control, see Discussion). The nucleotide sequences of miR-34a-5p, miR-155-5p, miR-186-5p, miR-200a-3p, and miR-200c-3p are identical in cows (*Bos taurus, bta*) and mice (*Mus musculus, mmu*) except for a uridine extension in *bta*-miR-200a compared with *mmu*-miR-200a-3p. Therefore, the species identifiers bta and mmu are omitted in the remainder of this article and murine entries are used to designate sequences of miRs.

RNA (2 μg) was purified from ceca and livers by using the miRNeasy mini kit (Qiagen) and reverse transcribed using the miScript II RT kit (Qiagen). The expression of miR-34a-5p, miR-155-5p, miR-186-5p, miR-200a-3p, and miR-200c-3p was measured by RT-qPCR in a CFX Connect Real-Time PCR Detection System (BioRad). We used the QuantiTect® SYBR® Green PCR kit (Qiagen) and its universal reverse primer, and miR-specific forward primers: 5′-TGGCAGTGTCTTAGCTGGTTGT-3′ for miR-34a-5p, 5′-TTAATGCTAATCGTGATAGGGGT-3′ for miR-155-5p, 5′-CAAAGAATTCTCCTTTTGGGCT-3′ for miR-186-5p, 5′-TAACACTGTCTGGTAACGATGTT-3′ for miR-200a-3p, and 5′-TAATACTGCCGGGTAATGATGGA-3′ for miR-200c-3p. Synthetic *Caenorhabditis elegans* miR-39 was used to normalize for the efficiency of RT-qPCR amplification.

Statistical analysis

Homogeneity of variances was assessed by Levene's test. The Shapiro–Wilk test was used to assess normality of distribution. Data from cytokine and PCR analyses were not normally distributed, or variances were heterogeneous or both. Therefore, these data were analyzed by using the nonparametric Mann–Whitney U test. Survival data were analyzed by the log-rank (Mantel–Cox) test. Histopathology data were analyzed by nonparametric permutation and multiple comparisons conducted by using the Bonferroni–Holm adjustment. All statistical analyses were conducted using R version 3.1.1 (The R Project for Statistical Computing), except that the log-rank test was conducted using Prism 7 (GraphPad Software Inc.). Data represent means $\pm$ SDs, except for survival curves which represent means $\pm$ SEMs. Differences were considered significant when $P < 0.05$.

Results

Effect of dietary treatment on gastrointestinal health and life span

Histopathological evaluation of cecal tissues revealed less severe intestinal lesions in ERS *Mdr1a*$^{-/-}$ mice than in ERD *Mdr1a*$^{-/-}$ mice. In particular, scores for gland hyperplasia and stromal collapse as well as the additive score were ≤57% lower in ERS *Mdr1a*$^{-/-}$ mice than in ERD *Mdr1a*$^{-/-}$ mice (**Figure 1**A, **Supplemental Table 1**). No differences in the degree of neutrophil/inflammatory cell infiltration or crypt hyperplasia were observed in the cecal tissues of any *Mdr1a*$^{-/-}$ mice in the study (Figure 1B, Supplemental Table 1). As expected, histopathological scores were significantly lower in *Mdr1a*$^{+/+}$ wild-type controls than in *Mdr1a*$^{-/-}$ mice, and diets did not affect cecal health in *Mdr1a*$^{+/+}$ controls. There was no effect of diet on survival or the severity of colonic lesions in *Mdr1a*$^{-/-}$ mice under the conservative experimental conditions in this study (see Discussion), except for lower scores of stromal collapse in colons from ERD *Mdr1a*$^{-/-}$ than from ERS *Mdr1a*$^{-/-}$ mice, and higher inflammation scores in colons from ERD *Mdr1a*$^{+/+}$ than from ERS *Mdr1a*$^{+/+}$ mice (**Supplemental Figure 2**, **Supplemental Table 2**). Subsequent studies focused on cecal tissues.

Effect of dietary treatment on cecal mRNA expression

Eighty-seven mRNAs were differentially expressed by ≥200% in the ceca of *Mdr1a*$^{-/-}$ mice fed ERS and ERD (**Figure 2**, **Supplemental Table 3**); 6 of the differentially expressed mRNAs are putative targets of miR-200a-3p. Gene ontology analysis implicated 16 of the differentially expressed genes in immune function and inflammation, e.g., antigen binding, regulation of B-cell activation, and immunoglobulin receptor binding (**Supplemental Figure 3**, **Supplemental Table 4**).

Effect of dietary treatment on the concentration of cytokines

The serum concentration of CXCL9 was approximately 35.0% $\pm$ 31.0% lower in ERS *Mdr1a*$^{-/-}$ mice than in ERD *Mdr1a*$^{-/-}$ mice (**Figure 3**). Dietary treatment had no effect on the serum concentrations of the other 31 cytokines and chemokines that were analyzed (**Supplemental Table 5**). CXCL9 serum concentrations were lower in *Mdr1a*$^{+/+}$ wild-type mice than in *Mdr1a*$^{-/-}$ mice (Figure 3).
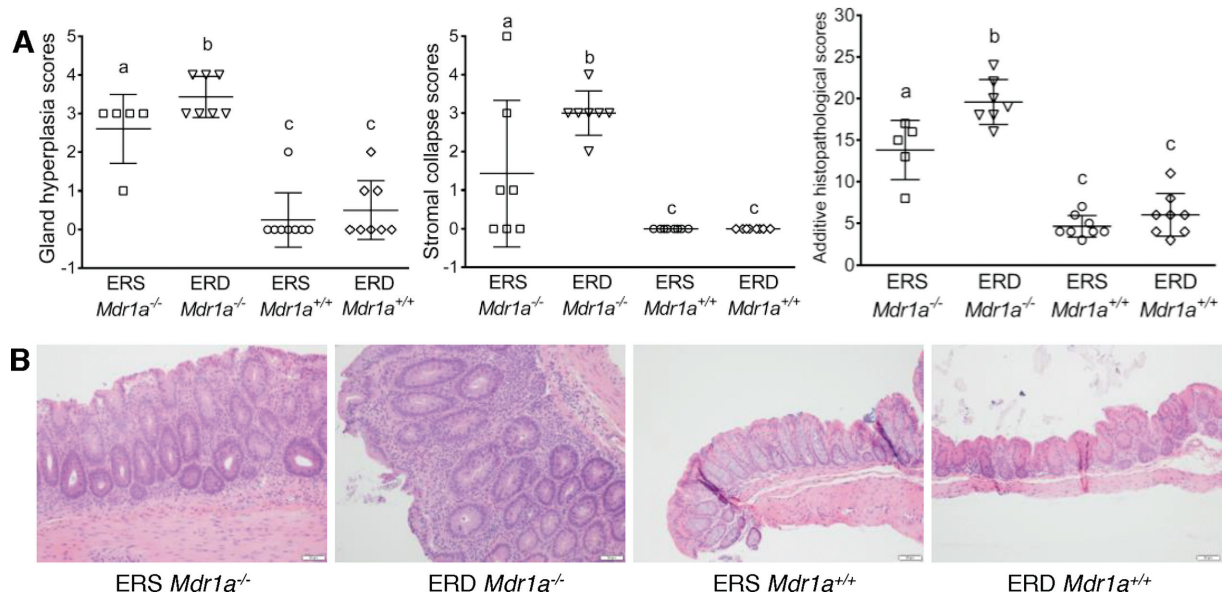
FIGURE 1 Histopathological assessment of cecal tissues harvested from $Mdr1a^{-/-}$ and $Mdr1a^{+/+}$ mice fed ERS and ERD diets. (A) Histopathological scores of ceca from $Mdr1a^{-/-}$ and $Mdr1a^{+/+}$ mice. Values are means ± SDs, n = 5–8 per treatment. Means without a common letter differ, $P < 0.05$. (B) Representative light microscopy images of hematoxylin and eosin–stained cecal tissues harvested from $Mdr1a^{-/-}$ and $Mdr1a^{+/+}$ mice (400-fold magnification). ERD, exosome- and RNA-depleted; ERS, exosome- and RNA-sufficient.

Effect of dietary treatment on miR expression in ceca and livers

Out of the 5 miRs that were tested, only the expression of miR-200a-3p was lower in the ceca from ERD $Mdr1a^{-/-}$ mice than from ERS $Mdr1a^{-/-}$ mice (**Figure 4**A); the expression of miR-200a-3p was 63% ± 9% lower in ERD $Mdr1a^{-/-}$ mice than in ERS $Mdr1a^{-/-}$ mice. In contrast, 4 of the 5 miRs were less abundant in livers from ERD $Mdr1a^{-/-}$ mice than from ERS $Mdr1a^{-/-}$ mice (Figure 4B); the expression of miR-200a-3p was 52% ± 12% lower in ERD $Mdr1a^{-/-}$ mice than in ERS $Mdr1a^{-/-}$ mice.

Discussion

This is the first study to our knowledge to demonstrate that depletion of dietary milk exosomes and their miR cargos exacerbates symptoms of IBD in mice. The effect sizes might appear to be modest, but they represent conservative estimates based on the following consideration: $Mdr1a^{-/-}$ mice were killed when their body weight loss exceeded 15% of their peak weight owing to stipulations by our Institutional Animal Care and Use Committee. We speculate that dietary treatment would have revealed larger differences in histopathology scores between $Mdr1a^{-/-}$ ERD mice and $Mdr1a^{-/-}$ ERS mice had the animals been monitored beyond the point of 15% weight loss compared with peak body weight. There is precedent for beneficial effects of milk exosomes and miR cargos in gastrointestinal health, e.g., porcine milk exosomes promote the proliferation of epithelial cells in the murine gastrointestinal tract (16).

This study also is the first that we know of to report that consumption of a miR-depleted diet causes a depletion of miRs in mouse tissues.

Previous studies reported a 61% decrease of plasma miR-29b and miR-200c concentrations in C57BL/6 mice fed ERD compared with ERS (6). Here, we report for the first time, as far as we know, that consumption of ERD is also associated with a drop of miR concentrations in tissues. It is reassuring to observe a comparable degree of depletion in circulation and tissues across 2 distinct murine genetic backgrounds. Our observation that the number of miRs affected by ERD feeding was greater in liver than in ceca is consistent with previous observations that miRs and their bovine exosome shells are absorbed in the upper intestine and accumulate primarily in the liver in mice (6, 11). Note that a crude estimate of exosome bioavailability in a previous study suggested that ≤75% of exosomes escape absorption and therefore may enter the large intestine and cecum (11). Depletion of miRs in tissues is consistent with phenotypes elicited by consumption of ERD, including increased tissue concentrations of purine metabolites and moderate loss of muscle grip strength compared with C57BL/6 mice fed ERS (18, 19). Previous reports of the bioavailability of dietary miRs, including those in milk, were met with skepticism in 4 studies (35–38). The limitations of these studies were discussed recently and include potential failure of overexpressed miR-30b to localize to exosomes, degradation of miRs in plasma samples, first passage elimination of miR-375 in the intestinal mucosa, and selection bias in bioinformatics protocols (39).

We chose miR-200a-3p as a model for assessing dietary depletion of miRs for the following reasons. First, miR-200a-3p has a sequence complementary to the 3′-UTR in Cxcl9 mRNA, which was the only cytokine differentially expressed in the ceca of ERD $Mdr1a^{-/-}$ and ERS $Mdr1a^{-/-}$ mice in this study. Second, murine miR-200a-3p and bovine miR-200a have identical nucleotide sequences with the exception of a uridine extension in a fraction of bovine miR-200a compared with murine miR-200a-3p, suggesting that bovine miR-200a-3p may regu-
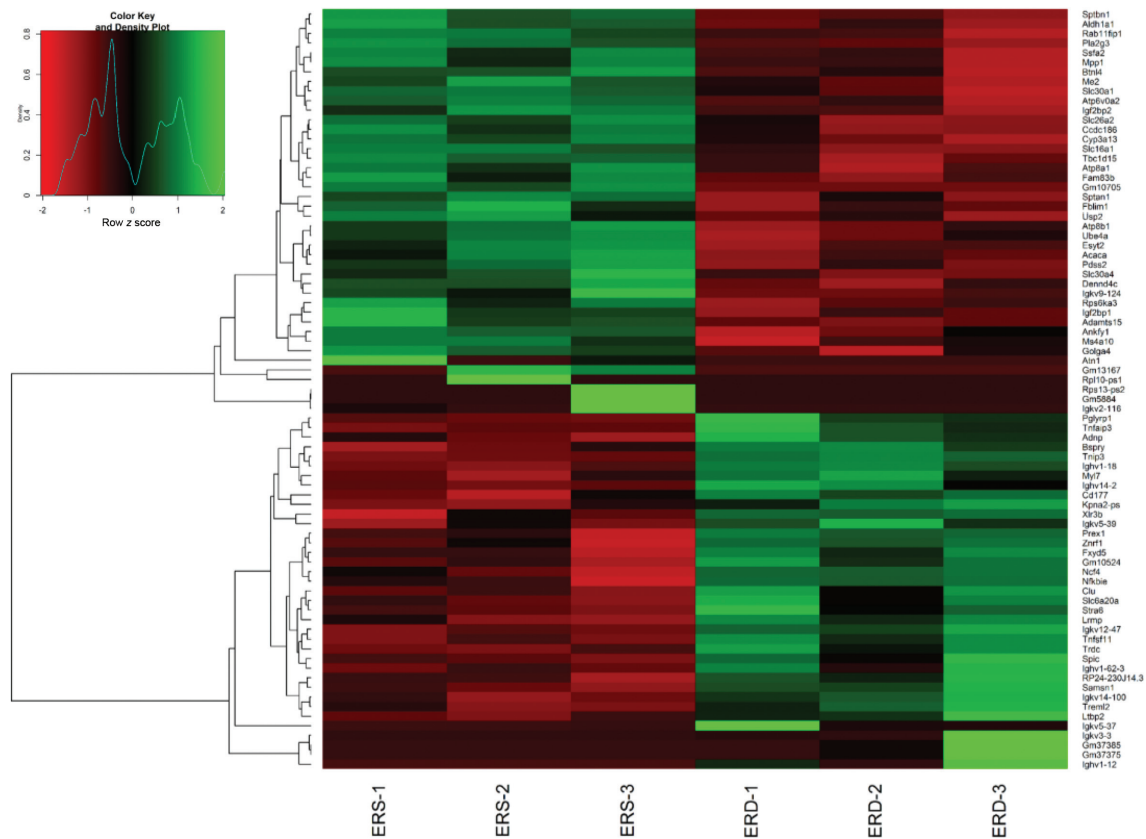
FIGURE 2 Differentially expressed mRNAs in ceca from ERS $Mdr1a^{-/-}$ and ERD $Mdr1a^{-/-}$ mice. Color-coded normalized expected counts for sequencing reads (red = low expression, green = high expression). $n = 3$. ERD, exosome- and RNA-depleted; ERS, exosome- and RNA-sufficient.

late murine mRNAs (40). Third, the content of miR-200a-3p in milk exosomes used to prepare ERD was completely depleted compared with that in ERS exosomes (30). Fourth, miR-200a-3p ranks among the 50 most abundant miRs in bovine milk exosomes (21).

We assessed 4 miRs in addition to miR-200a-3p in order to establish that dietary depletion of miRs in mice fed the ERD diet elicits a loss of miRs in tissues. We chose the miRs based on the following rationale. MiR-34a-5p and miR-155-5p were used as negative controls because consumption of 1.0 L milk did not cause a change in the plasma concentrations of these miRNAs in previous studies (41). We tested the expression of miR-186-5p because that miR was completely eliminated from milk exosomes during the preparation of the ERD diet (30). MiR-200c-3p was used as a positive control because the plasma concentrations of that miR were significantly lower in C57BL/6 mice fed ERD than in ERS controls in a previous study (6). The hepatic expression of miRs was largely consistent with previous observations, whereas interactions between the gut microbiome and milk exosomes might have caused minor deviations from the expected pattern in ceca (42).

We propose that the increased severity of intestinal inflammation in ERD $Mdr1a^{-/-}$ mice compared with ERS $Mdr1a^{-/-}$ mice is due to aberrant regulation of CXCL9 expression. CXCL9 is one of the ligands

of C-X-C chemokine receptor type 3, which has been implicated in the pathogenesis of IBD (43). Serum CXCL9 protein concentrations were lower in $Mdr1a^{-/-}$ ERS mice than in $Mdr1a^{-/-}$ ERD mice and Cxcl9 transcript levels were not different in ceca from the 2 diet groups. We explain this apparent disconnect by the low degree of complementarity between miR-200a-3p and its binding site in the 3′-UTR in Cxcl9 mRNA outside the seed region in miR-200a-3p. Complementarity is only 59% for miR-200a-3p and its binding site in the 3′-UTR in Cxcl9 mRNA, including the perfect match in the seed region. When sequence complementarity is low, miRs silence genes through preventing the translation of mRNA into protein rather than through causing mRNA degradation (44). Although depletion of miR-200a-3p in ceca is a plausible mechanism contributing to IBD in ERD $Mdr1a^{-/-}$ mice, we speculate that loss or gain of miRs other than miR-200a-3p also played a role in causing inflammation. For example, the 3′-UTR of Cxcl9 mRNA has putative binding sites for >500 miRs, and only 6 of the 87 mRNAs that were differentially expressed in the ceca from $Mdr1a^{-/-}$ mice fed ERD and ERS are putative targets of miR-200a-3p.

One might ask whether depletion of miR-200a-3p promotes the initiation or the progression of IBD. This study does not permit answering this question because dietary depletion of miRs did not alter the expression of CXCL9 protein in $Mdr1a^{+/+}$ mice, which are not genetically
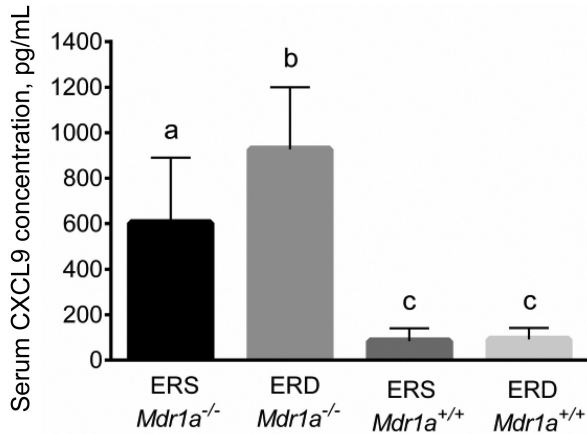
FIGURE 3 Serum CXCL9 concentrations in $Mdr1a^{-/-}$ and $Mdr1a^{+/+}$ mice fed ERS and ERD diets. Values are means ± SDs, $n = 8$. Means without a common letter differ, $P < 0.05$. CXCL, chemokine (C-X-C motif) ligand; ERD, exosome- and RNA-depleted; ERS, exosome- and RNA-sufficient.

predisposed to develop IBD. Also, there is no evidence in the published literature as to whether CXCL9 promotes initiation or progression or both.

Sixteen out of the 87 differentially expressed mRNAs in the ceca of $Mdr1a^{-/-}$ mice fed ERS or ERD are implicated in immune function and inflammation. Surprisingly, CXCL9 was the only serum chemokine that was affected by diet out of the 32 cytokines and chemokines that were tested. We propose that serum cytokine and chemokine concentrations may not necessarily be a good marker for their expression. Plasma concentrations are the net result of 2 opposing pathways: cytokine expression and secretion by donor cells into the plasma space, counteracted by cytokine receptor expression for cytokine uptake (clearance) by recipient cells from plasma. This being said, increases in cytokine expression might be canceled out by increased expression of membrane receptors

and, therefore, enhanced clearance. There is precedent for the expression of cytokines and their receptors having opposing effects on serum cytokine concentrations: biotin supplementation causes an increase in the expression of IL-2 and 1 of its receptors, IL-2 receptor-γ, leading to a net decrease of plasma IL-2 concentrations in response to biotin supplementation (45).

Studies of diet and IBD interactions are significant because an estimated 1.6 million people in the United States suffer from IBD (46). Approximately 70–80% of Crohn disease patients will require surgery at some point in their lives (47). The estimated health care costs of ulcerative colitis and Crohn disease are as high as $14.9 billion and $15.5 billion annually, respectively (48, 49). No cure has been identified and the only current treatment option is to ameliorate symptoms. There is precedent for our observation that milk exosomes slow inflammatory processes. Arntz et al. (50) reported that oral gavage of bovine milk exosomes ameliorated arthritis in IL-1 receptor antagonist$^{-/-}$ and DBA1/J mouse models. In contrast, we previously reported that variables of inflammation were higher in lungs of C57BL/6 mice fed ERS than in mice fed ERD when inflammatory responses were elicited by intranasal instillation of agricultural dust (51). This article suggests that symptoms of IBD were stronger in colons from ERS $Mdr1a^{-/-}$ mice than from ERD $Mdr1a^{-/-}$ mice, whereas symptoms were stronger in ceca from ERD $Mdr1a^{-/-}$ mice than from ERS $Mdr1a^{-/-}$ mice. We do not know how to reconcile the findings from these studies. We speculate that differences in tissue distribution of miRs and their exosome shells and the differential etiologies of IBD, arthritis, and asthma might play a causal role in explaining the detrimental effect of milk exosomes in asthma and their beneficial effects in IBD and arthritis (11).

We conclude that this study provided a rationale for further exploration of milk exosomes and their cargos in inflammation. Potentially fruitful lines of investigation include studies of exosome cargos other than miRs, including long noncoding RNAs, mRNAs, proteins, and lipids. In addition, it would be worthwhile to assess the interactions among dietary exosomes, the gut microbiome, and IBD. The gut microbiome has been implicated in IBD, although causal relations have not
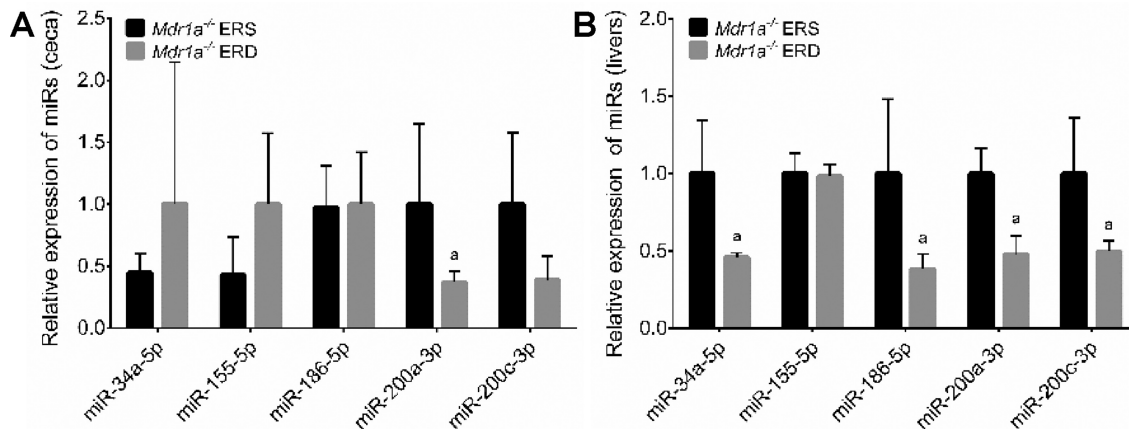

FIGURE 4 Expression of miRs in ceca (A) and livers (B) in $Mdr1a^{-/-}$ mice fed ERS and ERD diets. Values are means ± SDs, $n = 4$–8. ^{a}Significant difference from ERS, $P < 0.05$. ERD, exosome- and RNA-depleted; ERS, exosome- and RNA-sufficient; miR, microRNA.

been established (52). Our studies suggest that milk exosomes and their cargos have an effect on microbial communities in the murine gut (42).

Acknowledgments

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